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Improving Crop Resistance to Abiotic Stress

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Improving Crop Resistance to Abiotic Stress

Volume 1

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Abiotic stress tolerance QTLs mapped on the barley genome, excerpt, for further information see Fig 34.1.

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Foreword I

We are guests of green plants on this planet. Plants are a source of food, fiber, and materials for shelter. Ornamental plants contribute to our esthetic environment. Numerous plants are sources of pharmaceuticals. Our civilization developed progressively after the domestication of plants about 10 000 years ago. Since then plants were constantly improved through conscious and unconscious selection by ancient farmers for more than 9000 years. During the last century, crop improvement became a scientific endeavor after the rediscovery of Mendel's laws of inheritance. The science of genetics provided many additions to plant breeder's tool kit and major advances in food production were made. Green Revolution is a shining example of these advances. It has been possible to feed 6 billion of Earth's inhabitants.

Human population continues to increase unabated. It is estimated that there will be 9 billion people on this planet in 2050 and this will require doubling of food production. To meet this challenge, we must increase the yield potential of our food crops and close the yield gap. The average yield of most crops is about half their potential yield. For example, yield potential of rice is 10 ton ha^{-1}, but farmers on average harvest about 5 ton ha^{-1}. This yield gap is due to losses caused by biotic and abiotic stresses. Abiotic stresses include drought, submergence, salinity, and unfavorable temperatures.

Very little progress has been made in developing crops with tolerance to abiotic stresses through conventional breeding approaches. Breakthroughs in molecular biology and biotechnology have provided new tools such as molecular marker-aided selection (MAS) and genetic engineering. These technologies have opened new avenues for developing crops with tolerance to abiotic stresses.

Editors of this volume have done an admirable job of assembling a wealth of information on these new approaches for crop improvement. They have sought contributions from knowledgeable authors from all over the world. The number of crops included in the volume is comprehensive. These include grain, oil, fruits, vegetable, and ornamental crops and sugarcane, tea, tobacco, and cassava. Several chapters provide overview of latest advances in molecular biology such as genomics, transcriptomics, proteomics, and metabolomics, collectively called "omics." There is

an excellent chapter on the role of plant transporters in abiotic stress tolerance. The chapter on improving crop productivity under changing environment is a welcome addition in view of concerns about the impact of climate change on crop productivity. This comprehensive volume should prove useful for basic researchers, plant scientists, and students interested in crop improvement, as well as teachers.

I would like to congratulate the editors for their labor of love for preparing this valuable scientific resource.

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Foreword II

Together with other photosynthetic organisms, plants are the primary producers and the foundation of the global biogeochemical cycles that sustain terrestrial life. As such, plants are also the main biological resource for humans by providing food, feed, and various biomaterials such as oils, fibers, and wood. Taking into account population growth, urbanization, climate change, and the limitation of natural resources, global food security has become a strategic challenge just half a century after the "Green Revolution." There is a need for higher stability of yield to ensure global food security and repartitioning and lowering the prices of plant products. Moreover, the need to cut CO_2 emissions and the foreseeable end of the oil era makes the transition from conventional fossil fuels to alternative and renewable resources a priority, resulting in a growing demand for plant biomass for alternative energies and green chemistry.

Agriculture is also challenged by increasing urbanization and industrial pollution, resulting in the overexploitation of fossil resources, water, and arable land. Seventy percent of freshwater is used for irrigation, making water one of the most critical parameters in plant production. The predictions in climate change for this century are estimated to further negatively affect water supplies and agricultural productivity leading to the potential amplification of catastrophic incidents. Forty percent of the Earth's land surface is now used for agriculture. However, this area cannot be enlarged and instead, we foresee a reduction in arable land due to urbanization, pollution, and climate change in the next decades. If this was not enough, the world population will reach 9.2 billion by 2050, revealing that food production will have to double and farm productivity to increase by 1.75% each year.

In the face of these challenges, there is an urgent need to develop new crop lines that can perform better but under conditions of less water, less nutrient inputs, and by better withstanding abiotic and biotic stresses. This book, edited by Drs. Narendra Tuteja, Sarvajeet Singh Gill, Antonio F. Tiburcio, and Renu Tuteja, comes at the right time to tackle the problems plants face under abiotic stress conditions and will clearly be of major value for researchers and breeders. The editors have achieved to assemble a number of experts that share their knowledge in a very complementary

way. The volume thereby provides both an excellent overview and a detailed account of the field of plant abiotic stress response mechanisms. Importantly, the contributions range from established concepts in model plants to applied questions in specific crops. The book thereby will enlighten readers of various disciplines and at various levels, bridging text book knowledge to application.

Paris *Heribert Hirt*

Contents

Preface

World population is expected to increase from the present ~7 billion to ~10 billion by the year 2050; therefore, a critical question that needs to be asked is whether the rate of increase in crop yield would be sufficient to feed all the people. For example, the average world cereal yield will need to reach 5 ton ha^{-1} from its present 3 ton ha^{-1} to maintain population growth (FAO). On the other hand, the crops' productivity is continuously decreasing year by year due to the negative impact of various environmental stresses. The abiotic stress factors such as heat, cold, drought, salinity, wounding, heavy metal toxicity, excess light, flooding, high-speed wind, nutrient loss, anaerobic conditions and radiations are the key elements limiting agricultural productivity worldwide. For example, the continuous increase in only salt and water stress can lead to 50% loss of arable lands by the year 2050, which will lead to a significant decrease in crop production and ultimately will cause losses worth hundreds of millions of dollars each year. Therefore, increased water-use efficiency and salt tolerance are important challenges for agricultural production in an ever-decreasing area of arable land. Global warming and climate change indicate that in the future there may be more extreme weather events triggering flooding and extreme temperature. Managing risks from extreme flood and temperature events will be a crucial component of climate change adaptation. It is, therefore, of utmost importance to develop and implement techniques that enhance the crop productivity in the presence of continuous stress. Overall, the biotechnology and genetic engineering play important roles in innovative economy and is crucial in addressing global changes such as population growth and climate change. The transgenic approaches could be one of the fastest ways to produce GMOs that can tolerate the stress and can produce good yield in the presence of continuous stress. But the crops classified as GMO will have to undergo a costly authorization process, which involves extensive risk assessment for human and animal health and the environment. This is a lengthy process, and also the public acceptance for GMO products on the market is much lower compared to conventional or organic ones, which could be attributed to the nonawareness among the general public, media, and NGOs. However, the biotech crops could be as safe as conventional crops because in those countries where transgenic crops have been grown, there have been no verifiable reports of health or environmental harm (UN FAO). Overall, the improvement of

crop resistance to the stresses is very essential these days in order to cope with the upcoming problem of food security.

In this book, "Improving Crop Resistance to Abiotic Stress," we present a collection of 54 chapters written by more than 180 experts in the field of crop improvement and abiotic stress tolerance with special emphasis on crop plants. The book is divided into two volumes, each containing 27 articles. It is a timely contribution to a topic that is of eminent importance. The included chapters provide a state-of-the-art account of the information available on crop improvement and abiotic stress tolerance in crop plants. In this book, we present various approaches for improving crop resistance to abiotic stress.

Volume 1 containing Chapters 1–27 has been divided into two parts, "Part I Introduction to Plant Abiotic Stress Response" and "Part II Methods to Improve Plant Abiotic Stress Responses: Section II A Introductory Methods and Section II B Omics" and Volume II containing again 27 chapters, from 28 to 54, is further divided into "Part III Species-Specific Case Studies: Section III A Graminoids; Section III B Fruit and Vegetable Crops; Section III C Vegetable Crops: Solanaceae; Section III D Oil Crops Including Brassicas; and Section III E Other Crops." The Part I of Volume I deals with the understanding of commoneome operatives in response to various abiotic stresses in plants; industrial perspective of abiotic stress tolerance; ROS generation and scavenging under abiotic stress; salinity as major crop constraint; cold and abiotic stress signaling; sulfur dioxide toxicity and tolerance and excess soil phosphorus and remediation strategies, whereas Part II of the same volume covers various chapters under Section IIA that includes past, present, and future of genetically modified crops and translational biology approaches for abiotic stress tolerance. Section II B deals with the functional genomics of drought tolerance in crops; transcriptomic and metabolomic approaches for freezing tolerance and cold acclimation; overview of omics techniques in crop research and *Arabidopsis*; functional genomics and computational biology tools for gene discovery for abiotic stress tolerance; overview of transcriptomics and proteomics approaches for stress-responsive mechanisms; plant tissue culture and genetic transformation for crop improvement; systems-based molecular biology analysis of resurrection plants; molecular breeding for abiotic stress using halophytes; helicases for abiotic stress tolerance; transcription factors in general and MYB transcription factors for improving abiotic stress in plants; transporters in general and potassium and sodium transporters for improving abiotic stress tolerance in crop plants; fungus (*Piriformospora indica*) assisted abiotic stress tolerance; microRNA-mediated stress resistance in crop plants and polyamines in developing stress-resistant crops.

Volume II of the book starts with Part III "Species-Specific Case Studies: Section III A Graminoids," which uncovers the importance of various approaches such as functional genomics, omics, genomics-assisted breeding, physiological and molecular approaches for abiotic stress tolerance, and crop improvement in wheat, rice, maize, barley, sugarcane, and sorghum. Section III B covers different approaches like omics, grafting for improving crop productivity and abiotic stress tolerance of various vegetable crops such as chilli pepper, onion, soybean, chickpea, and peanut and fruit crops such as cassava. Section III C focuses on tomato, potato, and tobacco

for improving crop productivity and understanding the mechanism of abiotic stress tolerance. Section III D specifically deals with oil crops including Brassicas where various means to improve crop productivity and abiotic stress tolerance in oil crops such as sunflower, sesame, *Jatropha curcas*, and *Breassica* crop species including mustard have been included. Section III E includes genetic improvement approaches for drought tolerance in cotton with present status and research needs, and present status and strategies to improve abiotic stress tolerance in tea.

The editors and contributing authors trust that this book will provide a practical update on our knowledge of improving crop resistance in various crop plants and lead to new discussions and efforts to the use of various tools for the improvement of crop productivity.

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Part I
Introduction to Plant Abiotic Strees Response

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1
Understanding the "Commoneome" Operative in Plants in Response to Various Abiotic Stresses

Hemant R. Kushwaha, Sneh L. Singla-Pareek, Sudhir K. Sopory, and Ashwani Pareek

Environmental conditions do play a major role in the overall development and productivity of various crop species, but the exact relationship between the two has not been clearly defined. One of the major limiting factors in today's agriculture is salinity, which has not only led to loss of crop productivity but has also surmounted the economical loss. Various transgenic approaches have been undertaken to identify and establish the association between the stress tolerance of various crop plants and their environment. But limited success has been achieved because of the complex interplay of various genes and gene families in stress tolerance mechanism. Latest technological advancements such as genome sequencing and high-throughput expression profiling experiments have produced large amount of data, which can assist in elucidating stress responsive network of various genes and gene families related to stress tolerance. A newly established area commonly known as comparative genomics has laid the foundation for comparing various contrasting cultivars, in order to understand the mechanism of differential tolerance and susceptibility. A "commoneome" between various plant species can fill the gaps that have till now inhibited the knowledge of complex interplay of various genes. Earlier, commoneome between *Arabidopsis*, rice, and common ice plant has been analyzed, but in order to have a broader perspective, commoneome has been worked out from the barrier of monocot/dicot divide. Thus, this chapter would not only benefit the identification of newer gene families in plant species but also assist in hypothesizing various transgenic varieties with better stress tolerance.

1.1
Introduction

Plants, due to their sessile nature, often have to deal with diverse environmental stresses. Productivity of various crop plants is critically affected due to prevailing stress conditions [1]. Although poorly understood, the relationship between crop productivity and stress is of great biological, agricultural, and economic importance. One of the major stresses that limits the crop productivity is salinity, contributed largely by NaCl [2]. According to an estimate, about 8 million hectares of agricultural

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land in the world is exposed to salt stress conditions [3]. Despite the intensive technological advancements and management practices, modern-day agriculture is strongly influenced by salt stress. Therefore, an approach to increase the level of crop stress tolerance by activating a stress-responsive signal transduction pathway in transgenic plants seems to be a promising approach [4–7]. Owing to lack of precise knowledge about molecular and genetic basis of stress response, understanding the complex mechanism of the stress tolerance is still an arduous task.

Several processes in plants such as carbon metabolism, ion partitioning, energy metabolism, and growth have been observed to get affected by salt stress [8]. The mechanism of salt stress tolerance in various crop plants is hypothesized to be controlled by a large number of genes, which are further regulated by complex networks [9–11]. Hence, traditional breeding and marker-assisted breeding approaches have been used in the past, but they had limited success in terms of improving salt stress tolerance among various crop species. On the other hand, substantial efforts have been made using transgenic approach to understand, characterize, and improve salt stress tolerance, which also yielded limited success [12–15]. Many physiological and molecular biology studies carried out in recent past have generated many hypotheses by interpreting correlative evidence from many species based on biochemical and biophysical principles that govern stress tolerance [16]. Therefore, an analytical approach is required that can emphasize on the multigenicity of stress response and assist in developing crops tolerant to salt stress.

Genomics is one of the approaches considered to lay emphasis on the integrated analysis of stress-dependent behavior of the entire set of genes of a plant. It basically integrates traditional area of genetics analyzed with the help of bioinformatics tools and could possibly serve as a bridge between molecular biology and whole-plant physiology, agronomy, and crop breeding [11, 17]. The area of genomics has proven to be crucial in understanding the functional role of genes and their evolutionary history. It has been useful in identifying functionally important regions and deals with the study of complete genomes, particularly the set of techniques, analytical methods, and scientific questions related to the study of complete genomes.

In recent years, we have witnessed spectacular advances in functional genomics such as completion of the various genome sequencing [18, 19] and high-throughput expression profiling experiments [20] that have generated a large amount of data. This has led to the development of various computational approaches to analyze and predict unknown genes and gene families responsible for various responses. Also, with the assistance of computational genomics, genes responding to various environmental stimuli can be characterized and mapped on the whole genome, providing the essential foundation for more detailed analysis and thus elucidating the stress-responsive network of genes. Various attempts have been made earlier where genomics has laid the foundation for detailed analysis of various gene families performing diverse functions (Table 1.1).

Various specialized branches of genomics have now been used in order to develop a complete framework for understanding the complexity of stress response and tolerance. Classical genomics, which is related to the identification of various genes,

Table 1.1 List of gene families analyzed in various plant systems using genomics and expression profiling.

Gene Family	Function	Reference
CesA	Synthesis of wall	[21]
Lipocalins	Modulation of cell growth, metabolism, membrane biogenesis, and repair, induction of apoptosis, and environmental stress response	[22]
SAURs	Early auxin-responsive genes	[23]
Cysteine-rich antimicrobial peptides	Innate defense against pathogens	[24]
Calmodulin	Regulating cellular responses to stimuli by playing critical role as Ca^{2+} transducers	[25]
F-box	Critical for the controlled degradation of cellular proteins	[26]
Homeobox genes	Regulating plant growth and development	[27]

their structures, and localization on whole genomes can assist in developing various QTL maps and analyze linkage of salt stress-responsive genes. Functional genomics approach using various techniques such as microarray, SAGE, and so on can help in understanding the plethora of stress-responsive genes, while statistical genomics assists in analyzing the quantitative data obtained using intensive statistical methods. Comparative genomics is the one promising approach that is now-a-days used by a large number of research groups (Table 1.2), for understanding the mechanism of differential tolerance and susceptibility [28].

In our earlier study, we took a closer look at the transcriptome data obtained from various microarray analyses and thus reflecting the "commoneome" in *Arabidopsis*, rice, and common ice plant [36]. With the aim of underlining the commonality and differences in gene expression profiles, we have extended this study to other crop species, namely, maize, sorghum, and poplar and thus extended the commoneome in view of monocot/dicot divide. Furthermore, a general comment on the above-mentioned species related to their genome-related studies has also been made in order to shed light on the transcriptome analysis with respect to pre- and post-sequencing efforts.

1.2 Genomics-Based Studies in the Model Dicot Plants

1.2.1 Arabidopsis

Approximately two decades ago, *Arabidopsis thaliana*, a member of mustard family (Cruciferae or Brassicaceae), was accepted as a model plant for various plant biology

Table 1.2 List of some of the gene families analyzed using comparative genomics approach in various plant genera.

Gene family	Organism compared	Function	Reference
PDI	*Arabidopsis*, rice and maize	Formation of proper disulfide bonds during protein folding	[29]
PEBP	*Arabidopsis*, rice, maize, wheat, sorghum	Act on the control of flowering time	[30]
APx	*Arabidopsis* and rice	Catalyze the conversion of H_2O_2 to H_2O, using ascorbate as the specific electron donor in this enzymatic reaction	[31]
Dof	*Arabidopsis*, poplar, and rice	DNA binding with one finger domain transcription factor	[32]
TCS	*Arabidopsis* and rice	Signaling	[33]
Endo-β-mannase	*Arabidopsis*, poplar, and rice	Function in seed germination and other plant biological processes	[34]
CBS	*Arabidopsis* and rice	Probable role in salt stress response	[35]

studies, mainly due to its short generation time, small size, and a relatively small nuclear genome. The full-length 130 Mb sequence of *Arabidopsis* genome was made publicly available in 2000 [18]. Since then, *Arabidopsis* has come a long way in the area of plant biology research and has led to characterization of many plant genes and gene families. The observed synteny of *Arabidopsis* genome with other plant genomes such as rice, *Medicago*, and so on has assisted in the characterization of various stress-responsive genes, pathways, and processes in other plant species. Various genetic and molecular analyses of *Arabidopsis* mutants have shed light on various processes related to stress response and tolerance that were shadowed for long, in various plant species. Research groups around the world have been extensively involved in analyzing the plant system through *Arabidopsis*. Expression profiles pertaining to the physiological status of *Arabidopsis* such as time series data related to development, manipulative treatments, various stress treatments, and genetic intervention, obtained using microarray, have been made publicly available by *Arabidopsis* information resource (TAIR) (see www.arabidopsis.org) [37].

Transcriptome analysis using microarray data has revealed the relationships among stress-regulated transcripts and has enabled the prediction of their *cis*-regulatory elements [38, 39]. The correlation between expression profiles and the 5′ regulatory motifs of stress-regulated genes has been analyzed comprehensively [40]. In a recent analysis, osmotic stress-responsive genes have been identified in *Arabidopsis* [41]. Zeller *et al.* [42] have used whole-genome tiling arrays to analyze stress-induced changes with respect to salt, osmotic, cold, and heat stress, as well as ABA treatments, in the *Arabidopsis* transcriptome. With such a host of information

available on *Arabidopsis*, plant researchers can take advantage to develop a comparative view of the genome-based studies and contribute to engineering-improved crops with desirable and sustainable traits.

1.2.2 Common Ice Plant

One of the facultative halophytes, *Mesembryanthemum crystallinum*, known as common ice plant (because of its icy look due to enlarged bladder cells of leaf epidermis), has been accepted as another model plant for studying salinity stress responses at physiological, biochemical, and gene levels. Upon exposure to salinity, this facultative halophyte switches from C3 mode to CAM mode of photosynthesis. The other mechanism that helps common ice plant to adapt to salt stress condition involves synthesis and cytoplasmic accumulation of osmoprotective metabolites and accumulation of sodium in the vacuolar compartment [43]. Golldack and Dietz [44] suggested that the adaptation of the common ice plant to increased salinity is not because of its general and uniform cell response to stress, but because the response is a complex multicellular whole-plant response that depends on intercellular signaling processes. Comprehensive genomic analyses of salt stress-regulated genes have been performed in common ice plant [45]. Analysis shows that leaf tissues of well-watered and salinity-stressed common ice plant express ~15% more functionally unknown genes than the unstressed plant [46]. Recent analysis has established an extensive catalog of gene expression patterns in common ice plant that looks into the complex, transcriptional hierarchies that govern CAM-specific expression patterns [47].

1.2.3 Tomato

Tomato (*Solanum lycopersicum*), a member of Solanacae family, is one of the other major economic crops. The inherent features of tomato, such as diploidy, modestly sized genome (950 Mb), tolerance of inbreeding, amenability to genetic transformation, and availability of well-characterized genetic resources, makes it an important crop species for genetic and molecular research in Solanaceae [48]. In an initiative funded by National Science Foundation Plant Genome, EST sequences in tomato were made publicly available (http://www.tigr.org/tdb/tgi/lgi; http://www.sgn.cornell.edu) [48, 49]. According to an estimate, tomato genome has ~35,000 genes, considerably more than the 25,500 genes in the well-studied *Arabidopsis* genome. It has been predicted that tomato has diverged from the lineage leading to *Arabidopsis* approximately 150 million years ago (MYA). Through the comparative genomics approaches, it was observed that the majority of the tomato genes (70%) have significant matches to *Arabidopsis* genes, therefore, reflecting conserved gene functions [48]. Analysis of tomato genome, based on probing random cDNAs on genomic DNA gel blots, showed that ~47% of the tomato genes belong to multigene families [50]. In an analysis, correlated expression profile for 6758 genes across 25 different tomato tissues has been made and is compared to *Arabidopsis* and grape

tissues to identify differentially expressed and tissue-specific genes [51]. The cultivated tomato is essentially classified as a moderately salt-sensitive plant [52]. It has been considered that the salt stress response in tomato is regulated by a complex genetic mechanism [53]. Under salt stress conditions, tolerant genotypes of tomato maintain inner cellular osmotic status by accumulating higher content of inositol and sugars in their leaves [54]. Transcript profiling in tomato has shown that salt stress affects many pathways in the crop plant [55, 56]. Recently, a molecular mechanism for salt tolerance/sensitivity has been proposed using a comparative analysis between salt-treated and nontreated leaves of tomato [57].

1.3 Genomics-Based Studies in the Model Monocot Plants

1.3.1 Rice

Owing to the whole-genome sequencing and worldwide popularity, rice (*Oryza sativa*) is considered an important model crop among monocots for genomic analysis. For its small genome (430 Mb) and predicted high gene density, rice is considered an attractive target for cereal gene discovery efforts and genome sequence analysis [19]. The completion of the genome sequence of rice in 2005 has given plant biologist a platform to characterize various genes and genomes playing vital role in various stress responses and tolerance [58]. Even though rice is generally considered to be salt sensitive, there is huge genetic variation for salt tolerance at critical stages in the cultivated gene pool [59, 60]. Rice salt sensitivity varies considerably across cultivars, and this feature has been exploited to identify novel genes and proteins that contribute to salt stress tolerance [61]. Some traditional cultivars and landraces of rice are more tolerant toward various abiotic stresses. These tolerant varieties can be considered good source for the resistant traits [62]. Comparing the genetic variation in salt-sensitive and salt-tolerant varieties can aid in understanding the salt-tolerant behavior of the crop plant. Transcriptomics approaches have been used to identify differentially regulated rice genes in response to salt stress for shoots [2, 63–65] and roots [66] and by comparing rice with other cereals [67].

1.3.2 Maize

A member of Poaceae family, maize is one of the widely grown C4 crop with a high rate of photosynthesis. It is considered one of the major experimental crop plants because of its worldwide popularity and moderate genome size (~2400 Mb). Analysis shows that the substantial phenotypic variability of the maize crop plant is attributed to its molecular diversity at the genome level [68]. Various microarray experiments have been used to determine the gene-level expression and identify QTL associated

with transcript variation of coregulated genes under various environmental conditions. The gene expression profile in maize has also been used to study the effect of various abiotic stresses in the crop plant [69–72]. In order to understand the abiotic stress response and tolerance in maize, a web-based approach has been developed that keeps the updated compilation of various QTL maps and major genes associated with abiotic stress (http://www.plantstress.com/biotech/index.asp? Flag = 1). Xu *et al*. [68] have done a comprehensive analysis in maize.

1.3.3
Sorghum

Drought-tolerant sorghum (*Sorghum bicolor*) is considered one of the top cereal crops in the world, used not only for food but also as a biofuel. Due to its small genome size (~730 Mb), it is also accepted as a model plant for functional genomics of Saccharinae and other C4 grasses [73]. Under high-temperature conditions, sorghum improves its carbon assimilation by using its complex biochemical and morphological specializations [74]. Comparative genomic studies have shown the presence of similar gene families in sorghum to that in *Arabidopsis*, rice, and poplar (see Figure 1.1). Previous studies have identified genes and QTL related to abiotic stresses including postreproductive-stage drought tolerance [75–78]. Transcriptomics-related studies in sorghum have identified differentially expressed genes that play a major role in various responses to abiotic stress such as drought, salinity, and ABA [79]. Owing to its closeness to rice genome than any other major cereal crops with complex genomes and high levels of gene duplication, sorghum is considered one of the ideal candidates for genomic analysis.

1.4
Salt Stress-Related Transcriptome Changes Across Diverse Genera

Monocots and dicots have diverged from common ancestors between 12 and 20 MYA. Whole-genome sequencing projects have initiated the era of comparative

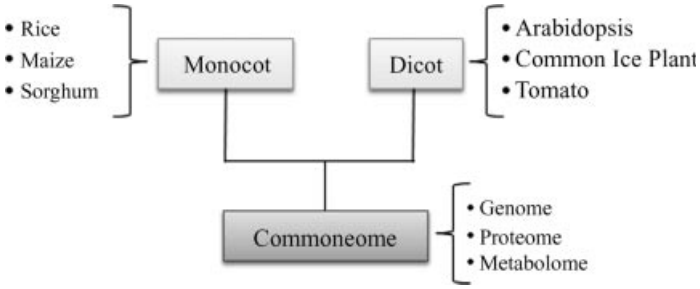

Figure 1.1 The "commoneome" approach – a possible route to identifying the "candidate genes" for stress tolerance in plants.

genomics, where the knowledge of key components of various stress-responsive genes and gene families is enhanced across monocot/dicot divide. Therefore, it has been accepted that the DNA sequence of the vascular plants has all the answers to the mystery of plant evolution and various responses that can be discovered only through comparative genomics. Stress response in plant is said to be multigenic in nature involving many genes and gene families. Our knowledge of complex stress response is ambiguous; therefore, a consolidated picture is still hidden. The identification of various convergent and divergent pathways between various stress responses can enhance the understanding of stress response in plants.

Earlier, we underlined the commonalities and differences in the gene expression profiles in three genera, namely, rice, *Arabidopsis*, and common ice plant by manually scoring the salinity-induced transcriptome changes [36]. Using the similar approach, we have now attempted to look beyond the monocot/dicot divide to elaborate our knowledge for salinity-induced transcriptomic changes. Here, we have emphasized on the genes that show alterations in species under consideration in response to salt stress. In this analysis, we have classified the gene expression as upregulated (depicted as $+$) and downregulated (depicted as $-$) under salt stress conditions. With the availability of extensive microarray expression data and widely popular species, *Arabidopsis* and rice, huge data sets are available for specific comparisons of the stress response. For other species, we relied on the microarray expression data (if available) or individual gene analysis carried out for those species.

1.5 Investigating the Salinity Stress-Related "Fingerprints"

The analysis of various model species in both monocots and dicots revealed a large repertoire of genes associated with salt stress response. These genes are further grouped into specific categories that are discussed in the following sections.

1.5.1 Stress Perception and Signaling

Crop plants often face varied environmental stresses. Therefore, in order to cope with these stresses, plants develop robust signaling mechanism for their perception. A complex cascade of molecules involved in processes ranging from stress perception to the final response in plants has been characterized that plays a vital role during the salt stress condition. These pathways include *S*alt *O*verly-*S*ensitive (SOS) pathway (SOS3-SOS2-SOS1) that regulates ion homeostasis under salt stress and results in Na^+ efflux and vacuolar compartmentation [80]; the calcium-dependent protein kinase (CDPK) pathway, which plays an important role in osmotic stress [80]; and the mitogen-activated protein (MAP) kinase pathway, which is important for counteracting both abiotic and biotic stresses [81]. In addition, two plant hormones, abscisic acid (ABA) and ethylene, also play an important role in abiotic stress response [82]. A recent study on the complex effects on root physiology in *Arabidopsis* has shown

upregulated ABA-marker genes in all cell layers of the roots, which suggests that ABA might primarily mediate semiubiquitous transcriptional responses to salt stress [83]. The overexpression of mouse calcineurin gene has led to higher salt tolerance in rice [40]. Wang *et al.* [84] identified a putative maize calcineurin B-like (CBL) gene that serves a function in salt stress-elicited calcium signaling and thus in tolerance to salinity. Guanylate kinase (GK), which encodes an enzyme important for the biosynthesis of nucleotides [85], and is hypothesized to be an important enzyme that is fundamental to second-messenger signal transduction pathways [86], has been characterized in three plant species, namely, *Arabidopsis*, lily, and tobacco [85, 87]. In another analysis, *AGK-1* and *AGK-2* were shown to be constitutively expressed in all tissues of *Arabidopsis*, but their transcription levels were found to be highest in roots [88]. A recent analysis has shown that overexpression of protein kinase SAPK4 in rice conferred increased tolerance to salt stress at the seedling stage and in mature plants [89].

1.5.2
Gene Regulation

Several regulatory molecules such as transcription factors (TFs) have been characterized that regulate gene expression under salt stress conditions including different classes of DNA binding proteins such as dehydration response element/C-repeat, Myb, and Myc proteins and proteins containing bZIP, Zn finger, or AP2 domains [2, 65, 66, 90–93]. The analysis of intronless *OSISAP1* gene that encodes for zinc finger protein shows that this gene gets induced in response to high levels of exposure to salt stress [90]. Zn finger protein Zat12 that plays a major role in metabolism of reactive oxygen species (ROS) as well as biotic and abiotic stress condition has been characterized in *Arabidopsis* [94]. Past studies have shown the role of C_2H_2-type zinc finger protein regulating stomatal aperture, thus playing a major role in salt and drought stress tolerance in rice [95–98]. Similar analysis shows that a receptor-like protein kinase and MYC-related DNA binding transcription factor is induced rapidly by high salt concentration [99, 100]. Another analysis shows that specific CaM isoform mediates salt-induced Ca^{2+} signaling through the activation of an MYB transcriptional activator, thereby resulting in salt tolerance in plants [101]. Another class of transcription factor known as NAC (NAM, ATAF, and CUC) family, which is one of the largest plant transcription factor families, was observed to be involved in diverse plant functions, and its overexpression in rice enhances drought resistance and salt tolerance in rice [97, 102]. Transgenic rice plants overexpressing one of the members of NAC gene family, *ONAC045*, showed enhanced drought and salt tolerance, indicating that it plays an important role in abiotic stress [103]. A subgroup of AP2/EREBP transcription factors known as dehydration-responsive element binding (DREB) proteins were found to play important role in plant response and tolerance to various abiotic stresses [104, 105]. Results have shown that some of the DREB genes were observed to be involved in both ABA signaling and stress-responsive pathways [106]. Also, it was observed that some DREB transcription

factors activated by ABA mediate their downstream gene expression, thus helping plants in stress tolerance [107–109].

1.6 Proteins Related to General Metabolism

Under salt stress conditions, several metabolic pathways in plants also get affected. Analysis of early salt stress-responsive genes in tomato root using suppression subtractive hybridization and microarray analysis identified genes involved in various metabolic pathways. Some of the genes involved in nitrogen fixation such as nitrate reductase, phenylalanine ammonia-lyase, (PAL), glutamine synthetase, and asparagine synthetase were found significantly upregulated during the salt stress treatment, while genes involved in methionine biosynthesis were found to be downregulated by the treatment [55]. Salinity stress enhances carbohydrate accumulation in tomato and leads to its movement from leaves to its fruit during early fruit development [110]. Salt stress (in roots of *Arabidopsis*) has shown to downregulate the enzymes involved in lignification, as well as the potentially cell wall-related lipid transfer proteins (LTPs). Interestingly, energy-evolving pathways were also found downregulated, and it is hypothesized that this may serve to conserve energy and limit growth [111, 112]. Many transporter proteins such as ABC (ATP binding cassette) transporter, LeOPT1-like transporters, and MATE-like (multiantimicrobial extrusion) efflux carriers were found to be induced by salt stress treatment [111]. Some of the cell wall-related families such as expansin and xylogulcanases were found to be induced by salt stress treatment that is also observed in response to osmotic stress in some species in order to increase the cell wall flexibility [111, 113]. In rice, genes involved in photosynthesis were also found to be downregulated upon salt treatment [114].

1.7 Stress-Induced Proteins with Some Protective Functions

Salt stress in crop plants induces various protective and adaptive responses in order to minimize the effect of stress. The dehydrin proteins that have been hypothesized to be associated with membrane and protein stability, metal scavenging, and suppression of ROS-induced damage were found to be upregulated in sorghum upon exposure to salinity. Salt treatment to the leaves of tomato plant led to the induction of ferredoxin thioredoxin reductase protein that plays a major role in maintaining redox status in plants [56, 57]. Similar response was also observed with LEA proteins to salt stress [79, 115–117]. In rice, senescence-related genes such as aspartic proteinase, ClpC protease, NAD-dependent malate dehydrogenase, and polyubiquitin were found upregulated in response to salt stress [114]. The genes involved in cell defense and detoxification such as glutathione reductase, dehydroascorbate reductase, and phospholipid hydroperoxide glutathione peroxidase

were found upregulated during the salt stress treatment [114]. Analysis has shown that salt stress induces oxidative stress in plants, thus leading to the accumulation of H_2O_2 that acts as a signal for triggering the cell defense mechanism [118, 119]. Several heat shock proteins (HSPs) were observed to play a crucial role in plant stress response by reestablishing normal protein conformations and thus maintain cellular homeostasis [120]. Overexpression of DnaK1 from halotolerant *Cyanobacterium aphanothece* in transgenic tobacco showed increased tolerance to salt stress [121].

1.8
Proteins Related to Maintenance of Osmotic Homeostasis

It is well established that salt stress in plants leads to disruption of cellular, ionic, and osmotic homeostasis [122]. Thus, a plant often tends to maintain homeostasis at every level to survive the stress environment. Analysis shows that various transmembrane transport proteins play a curial role in ionic and osmotic homeostasis under salt environments [123]. Transmembrane proteins such as sodium ATPase (PpENA1 and PpENA2), vacuolar H^+-ATPase, PpSHP1 and PpSHP2, chloride channel protein, and ABC transporters play important role in osmotic homeostasis [124–127]. Extensive studies have been performed on V-ATPase to study its response to the salinity stress environment [128, 129]. Microarray analysis of transcripts in *Populus euphratica* under salt stress showed upregulation of genes assisting in maintaining osmotic homeostasis such as magnesium transporter-like protein, syntaxin-like protein, seed imbibition protein, plasma membrane intrinsic protein PY-PIP2-1, and aquaporin. Analysis of barley microarray transcripts revealed an increase in the level of Δ-pyrroline-5-carboxylase synthase (P5CS), which is a rate-limiting enzyme for accumulation of proline in plants [62, 130]. Osmoprotectants such as glycine betaine and trehalose (which act by stabilizing quaternary structures of proteins and highly ordered states of membranes) were also found upregulated under salinity stress [62]. Interestingly, it was observed that transgenic rice overproducing glycine betaine and trehalose accumulated fewer Na^+ ions and maintained K^+ uptake [131, 132].

1.9
Protein with Unknown Function

Whole-genome sequencing projects in plants have generated vast knowledge about genes and gene families. The next big challenge after their identification is characterizing them and further associating them with probable functions. In *Arabidopsis*, identification of more than 5000 unknown proteins have led to the hypothesis of their probable role in various pathways and networks that might be playing major role in various abiotic stress tolerance [133, 134]. The unknown proteins were named as

proteins with obscure features (POFs) and the proteins that contained at least one previously defined domain or motif were named as proteins with defined features (PDF) [133, 135]. Recently, one of these PDF, a CBS domain-containing protein family, has been characterized in rice and *Arabidopsis* and has been hypothesized to play a major role in abiotic stress tolerance and development [35]. Another study in *Arabidopsis* has established the role of some of the unknown proteins in oxidative stress tolerance by using transgenic approach [136, 137]. A large number of unknown protein coding genes were found to be regulated in microarray analysis under salt stress in tomato that needs further analysis [55].

1.10 Analysis of Stress Transcriptome from other Plant Species

Since the inception of the idea to study the complex plant stress tolerance mechanism using whole-genome microarray, extensive analysis on various plant species has been performed. Analysis of plants living under extreme saline conditions has extended the knowledge base that has helped in understanding the salt stress behavior of plants. Recently, salt-responsive genes have been identified in genome-wide analysis using cDNA microarray in common wheat [138]. It was observed that genes encoding various ion transporters and osmotic regulators were upregulated in salt stress treatment. Analysis has shown the upregulation of genes belonging to various gene families such as AP2/EREBP, MYB, NAC, and WRKY. Nemoto and Sasakuma [139] have identified and analyzed early salt stress-responding genes (WESR1-4) in common wheat (*Triticum aestivum* L.).

Medicago tranculata is one of the model plant species among dicots being used for genomic analysis owing to its small, diploid genome and has short generation time, self-fertility, and high transformation efficiency. It is considered to be closely related to important forage legume, alfalfa, and can serve as a model organism for soybean and other economically important legumes [140–143]. A recent analysis in *Medicago* has characterized ERF gene family members that were also characterized in rice and *Arabidopsis* [144]. Members of ERF gene family encode transcriptional regulators with a variety of functions involved in the developmental and physiological processes in plants and were found to play a crucial role in nodulation and in early Nod factor signaling in *Medicago* [145]. In an analysis, two genotypes of *Medicago* were compared and analyzed using macroarray of 384 genes linked to salt stress and recovery responses in roots [146, 147]. Gruber *et al*. [148] have identified several transcription factor genes in the root region that not only respond to the salt stress but are also induced by osmotic, heat, and cold stress. This analysis shows that these TF genes not only are the part of general stress response of root apexes but also are strongly induced under high salt condition, suggesting the complex interlinking of these TFs in the root region. Recently, a database (TRUNCATULIX) was made available that integrates the sequence, annotation, and gene expression data from several *M. truncatula* databases [149] (see http://lily.cebitec.uni-bielefeld.de/truncatulix/app). Thus, with the kind of analysis available for this species, the understanding of the stress tolerance and response will get further elaborated.

One of the halophyte commonly known as mangrove plant (*Bruguiera gymnorhiza*) has also been studied to understand the salt stress behavior of the plants. The identification of various mechanisms such as detoxification of ROS by superoxide dismutase [150], osmotic adjustments via sucrose biosynthesis [151], and increased total amino acid pool, especially proline [152] and polyphenol [153], has furthered our understanding of tolerance to salt stress in mangrove plant. Wonga *et al*. [154] have elaborated on the genes playing a major role in salt stress tolerance in mangrove plant using subtractive hybridization and bacterial functional screening. Tada and Kashimura [155] have performed the proteome analysis of the main and lateral roots and the leaves in salt-treated mangrove plant *B. gymnorhiza* and have observed enhanced expression of FBP aldolase in the main root of the salt-treated mangrove plant, which leads to osmolyte production and contributes to stress tolerance.

Widely grown for forage purpose and as a grain crop, *Hordeum vulgare* L., commonly known as barley, has been analyzed for the salt stress tolerance trait, as it is one of the salt-tolerant crops. Characterization of salinity stress in barley has revealed the involvement of multiple genes that are responsive to salinity stress using microarray and differential display [66, 156–159]. Analysis done by Walia *et al*. [62] showed that a large number of abiotic stress (heat, drought, and low temperature)-related genes were also found to be responsive to salinity stress in barley.

Potato, another economically important and moderately salt-sensitive crop, is also studied for its response under salt stress condition. Salt stress treatment of potato revealed increased activation of antioxidant enzyme and accumulation of proline as its response [160–163]. Various transcription factors, signal transduction factors, and HSPs were found to be associated with abiotic stress response in transcript profiling of potato [164]. The overexpression of proline production enzyme, Δ-pyrroline-5-carboxylate synthetase, has been found to play an important role in salt stress tolerance [165]. Proteome analysis has revealed the photosynthesis-related proteins and those related to protein synthesis were downregulated, whereas osmotin-like proteins, HSP, and calreticulin proteins were upregulated under salt stress conditions [166] (see Table 1.3).

Grape (*Vitis vinifera*) is another economically important crop plant being studied for salt stress response as most of the researchers have ranked it moderately sensitive to salinity stress [167–170]. In a recent study, it was observed that grapevine growth was more sensitive to water deficit than to an equivalent salinity level suggesting that the salt uptake contributed to osmotic adjustment and thus facilitated water uptake and growth in young shoots [171]. Increased transcript accumulation of photorespiratory enzymes, glycolate oxidase and catalase, in the peroxisome shows the effect of salinity on photorespiration [171. Also, a large number of transcripts involved in ABA metabolism or responsive to ABA were enhanced due to saline conditions. Recently, a database has been developed that provides genomic resource for grape genome annotation and gene function (http://cropdisease.ars.usda.gov/vitis_at/main-page.htm) [172].

Thellungiella halophila, a close relative of *A. thaliana*, has also been adapted as a model crop for analysis of abiotic stress tolerance in plants [173]. *Thellungiella* has been widely characterized as an extremophile, known for its extreme tolerance to a variety of abiotic stresses such as low humidity, freezing, and high salinity [14, 173,

Table 1.3 Up- and downregulated genes in monocots (rice, maize, and sorghum) and dicots (*Arabidopsis*, common ice plant, and tomato) in response to salinity stress.

Gene notation	Monocots			Dicots		
	Rice	Maize	Sorghum	*Arabidopsis*	Common ice plant	Tomato
Stress perception and signaling						
ABA- and stress-induced protein	+	+	+	+		
S-adenosylmethionine decarboxylase 2	+	+	+	+		+
Auxin regulated protein	+	+	+	+		
Calcium-dependent protein kinase	+	+		+	+	+
Calcineurin-like phosphatase	+	+		+		+
Calcium binding EF-hand protein	+			+		+
Gibberllic acid-induced gene	+			+	+	+
Lectin, lectin protein kinase	+			+		
Protein phosphatase 2C	+			+	+	+
Receptor kinase-like protein	+	+	+	+	+	+
Ser/Thr kinase-like protein	+	+	+	+	+	+
Stress regulation						
AP2 domain-containing transcription factor	+			+		+
CC-NBS-LRR resistance protein mla13	+	+	+	+		
Myb-like DNA binding domain	+	+	+	+		−
NAC-type DNA binding protein	+	+	+	+		+
Translation initiation factor	+			+		−
Zinc finger protein	+	+	+	+	+	+
bZIP DNA binding protein	+	+	+	+		−
General metabolism						
Aldehyde dehydrogenase	+		+	+		
Anthocyanin biosynthesis	+			+		
Ascorbate peroxidase, cystolic type	+			+		
Chlorophyll a–b binding protein	+			+		
Cytochrome P450 monooxygenase	+	+		+	+	+
Esterase/lipase/thioesterase-like protein	+			+		
Expansin, putative	+			+		+
Galactosidase	+			+		
Galactinol-raffinose galacto-syltransferase, galactinol synthase	+			+	+	

Table 1.3 (*Continued*)

Gene notation	Monocots			Dicots		
	Rice	Maize	Sorghum	*Arabidopsis*	Common ice plant	Tomato
β-glucosidase homologue	+			+	+	
Glutamate receptor family protein, glutamate synthase	+	+	+	+	−	−
Glycosyl transferase	+			+		
1,4-hydroxylphenylpyruvate dioxygenase	+	+		+		
Ribosomal protein	+	+	+	+	+	
Zeaxanthin epoxidase	+			+		
Protective function						
Acidic endochitinase	+			+	+	
Ankyrin repeat family protein	+			+		
Cold-regulated protein, cor15a	+	+		+	+	
Dehydrin, DREB subfamily	+	+	+	+		
β 1,3-glucanase	+			+		
Glutathione S-transferase homologue	+	+		+	+	+
Heat shock protein	+	+	+	+	+	+
LEA protein	+		+	+	+	
Lipooxygenase	+	+	+	+		
Metallothionein-like protein	+		+	+		
Papain cystein protease	+			+		
Pathogen-responsive dioxygenase	+			+		
Peroxidase-1	+	+	+	+	+	+
Protease inhibitor	+			+	+	
Subtilisin-chymotrypsin inhibitor 2	+			+		
Thioredoxin, thioredoxin reductase	+			+		−
Xyloglucan endotransglycosylase	+		+	+		
Osmotic homeostasis						
ABC transporter family	+	+	+	+	+	−
F-box family protein	+			+		
Ion transporter, Na/H	+	+		+	+	+
MATE efflux family protein	+			+		
Pyrroline 5-carboxylase synthetase	+			+		
Sugar transporter	+	+	+	+	+	+
Trehalose 6-phosphate phosphatase	+			+		

(*Continued*)

Table 1.3 (*Continued*)

Gene notation	Monocots			Dicots		
	Rice	Maize	Sorghum	*Arabidopsis*	Common ice plant	Tomato
UDP-glucose 4-epimerase	+		+	+		
V-type ATPase	+			+	+	+
Water channel protein	+			+	+	
Unknown function						
Glycine/serine-rich protein	+			+		
O-methyltransferase	+		−	+	+	
Others						
Adenine phosphoribosyl-transferase form 3	−			−		
Water channel protein (WCP-III)	−			−		
A-tubulin	−			−		
TMK (gibberellic acid induced)	−			−	−	
Peroxidase ATP19a	−			−	−	−
Putative translation initiation factor eIF-2Ba	−	−		−		−

Only those candidates have been selected that show a response common in at least two of the salinity-related transcriptome analyses. This analysis included both salinity upregulated (+) and downregulated (−) genes. Artificial grouping of these genes has also been done to reflect their possible physiological role(s).

174]. Gong *et al*. [175] observed activation of additional pathways that led to increase in metabolites in comparison to the pathways activated in *Arabidopsis* under salt stress. *Thellungiella* was observed to possess novel stress-relevant genes and also maintain higher expression of certain genes related to salt stress [175, 176]. In response to salt stress, proline levels were observed to have increased in *Thellungiella*, higher than that of *Arabidopsis* [174, 177–179]. Transcriptome analysis of the salinity-stressed plants of the Yukon ecotype of *Thellungiella* revealed a stress-specific response of the plant [180]. Knockout analysis of SOS1 gene by RNAi transformation method has shown the importance of SOS pathway in *Thellungiella* [181, 182]. Over the years, several cDNA libraries have been constructed from *Thellungiella* plants grown under various stress conditions, which were used for various comparative analyses [180, 183, 184]. Similar to *Thellugiella*, *Porteresia coarctata* is also considered a comparable halophytic model crop plant of *Oryza* spp. due to its salt-tolerant characterstics. A large number of genes related to the salt-stress tolerance in *Porteresia* have been isolated and characterized [185]. Recently, Sengupta and Majumder [186] took an initiative of proteomic analysis of *Porteresia* under salt stress. It has been considered that the analysis of physiological and metabolic

adaptation in *Porteresia* in comparison to *Oryza* spp. will provide finer details of the evolved salt stress tolerance mechanism.

1.11 Conclusions

With advancing technologies, the urge to understand and unravel the mystery of complex genomic response to various stresses in crop plants has led to the concussion in the existing knowledge. Till now we have information regarding the stress response in plants in the form of tessellating pieces. Therefore, these pieces need to be assembled to draw a complete picture (Figure 1.2). We have attempted to solve the jigsaw puzzle by elaborating on the "commoneome – common gene expression in various plant species" under salt stress condition. This attempt is rather complex as it is observed that various plants (monocots/dicots) seem to have varied gene expression in species-specific manner, though the response to the salt stress in terms of respective gene expression looks more or less similar.

Comparison of various plant species has helped not only in understanding their evolution, especially convergence and divergence, but also in getting valuable insights into their abiotic stress tolerance. Genome projects such as whole-genome

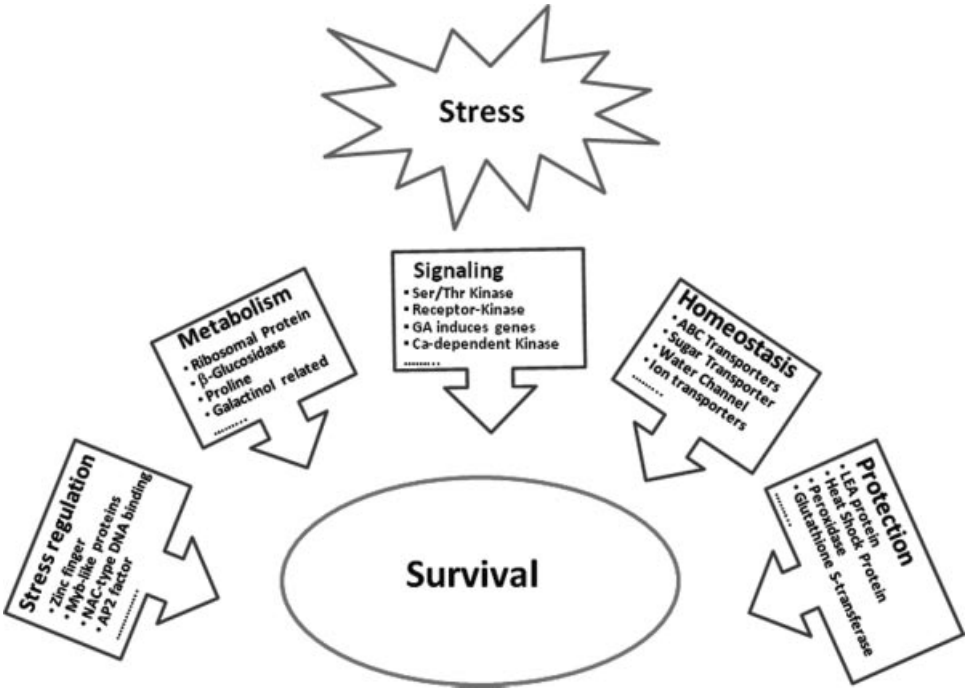

Figure 1.2 An overview of the role played by salinity-related transcriptome that assists plant survival under stress.

sequencing and transcript profiling under various conditions in *Arabidopsis*, rice, maize, and other plant species has provided basic knowledge bank for comparative genomic approaches. Availability of ESTs, microarrays, clusters of similarly expressing genes, molecular markers, mutants, and QTL maps in these plant species has ameliorated the current dogma of stress biology.

It is now widely accepted that salinity is one of the biggest problems gradually affecting the food crop production around the world. In order to develop crops that are able to tolerate salt stress, one needs to understand the effects of salt stress on crop plants that also vary from crop to crop. Approaches such as commoneome can help in developing a better understanding of such responses. In the earlier study, we attempted to compare three model crop plants, namely, rice, *Arabidopsis*, and common ice plant [36]. In this study, we have elaborated on the commoneome further, by looking at the salt stress response beyond the monocot/dicot divide. It is learnt that gene and gene families often play a role in multiple pathways and processes; therefore, they cannot be categorized specifically on one single function. Hence, these genes and gene families were arbitrarily clustered into specific tasks they majorly participate in such as signaling, regulation, metabolism, and so on.

Signaling plays a major role in the inception of the stress and hence the plants overall response. Several pathways, processes, and molecules play an important role in signaling mechanism. One of the widely studied pathways, the SOS pathway, is known to regulate ion homeostasis under salt stress. Other known pathways, namely, CDPK pathway and MAP kinase pathway, play an important role in osmotic stress. Plant hormones such as ABA and ethylene play a role in abiotic stress response. Several transcription factors also help in salt stress response by regulating various genes' expressions. Some of these DNA binding proteins that play important role are DREB, Myb, and Myc proteins, bZIP, Zn finger, AP2 domain proteins, and NAC family proteins that have been studied in various plants. Salt stress is known to affect many gene transcripts involved in metabolism, protein fate, transport, transcription, and cellular defense and also photosynthesis and photorespiration.

Complexity of salt stress response is a major cause of incomplete understanding of plant response to stress. A large number of genes and gene families are still either unrecognized or unknown, which might be playing a role in its response. A large number of pathways are still to be deciphered that can shed light on and enhance our knowledge of the plants adaptability to various stress conditions. There has been some development in this regard as glyoxalase pathway (which has been proposed to be involved in various essential functions in animal system) has been shown to have a direct correlation with enhancement in salinity tolerance in plants [187, 188]). However, the members of this important gene family are not picked up in our commoneome analysis. Thus, we can safely conclude that there may be still numerous genes "unidentified" and/or "uncharacterized" from various databases that are labeled "unknown," but may have important contribution to a given stress response in plants. Comparative analysis of transcriptomes and metabolomes between salt-sensitive and salt-tolerant lines can also help in identifying "candidate" genes. With integrative genomic approaches, we can hope to fill these gaps and develop a better crop that can survive under harsh environment conditions.

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References

1 Boyer, J.S. (1982) *Science*, **218**, 443–448.
2 Sahi, C., Agarwal, M., Reddy, M.K. *et al.* (2003) *Theor. Appl. Genet.*, **106**, 620–628.
3 Munns, R. (2005) *New Phytol.*, **167**, 645–663.
4 Cushman, J.C. and Bohnert, H.J. (2000) *Curr. Opin. Plant Biol.*, **3**, 117–124.
5 Kasuga, M., Liu, Q., Miura, S. *et al.* (1999) *Nat. Biotechnol.*, **17**, 287–291.
6 Kovtun, Y., Chiu, W.L., Tena, G. *et al.* (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 2940–2945.
7 Umezawa, T., Yoshida, R., Maruyama, K. *et al.* (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 17306–17311.
8 Hasegawa, P.M., Bressan, R.A., Zhu, J.K. *et al.* (2000) *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **51**, 463–499.
9 Bartels, D. and Sunkar, R. (2005) *Crit. Rev. Plant Sci.*, **24**, 23–58.
10 Chinnusamy, V., Jagendorf, A., and Zhu, J.K. (2005) *Crop Sci.*, **45**, 437–448.
11 Sahi, C., Singh, A., Blumwald, E. *et al.* (2006) *Physiol. Plant*, **127**, 1–9.
12 Singla-Pareek, S.L., Reddy, M.K., and Sopory, S.K. (2001) *Proc. Ind. Natl. Sci. Acad.*, **B67** (5), 265–284.
13 Grover, A., Aggarwal, P.K., Kapoor, A. *et al.* (2003) *Curr. Sci.*, **84**, 355–367.
14 Wang, W., Vinocur, B., and Altman, A. (2003) *Planta*, **218**, 1–14.
15 Yamaguchi, T. and Blumwald, E. (2005) *Trends Plant Sci.*, **10**, 615–620.
16 Levitt, J. (1980) *Responses of Plant to Environmental Stress Chilling, Freezing, and High Temperature Stresses*, 2nd edn, Academic Press, New York.
17 Bohnert, H.J., Gong, Q., Li, P. *et al.* (2006) *Curr. Opin. Plant Biol.*, **9**, 180–188.
18 AGI (Arabidopsis Genome Initiative) (2000) *Nature*, **408**, 796–815.
19 Goff, S.A., Ricke, D., Lan, T.-H. *et al.* (2002) *Science*, **296**, 92–100.
20 Schena, M., Shalon, D., Davis, R.W. *et al.* (1995) *Science*, **270**, 467–470.
21 Burton, R.A., Shirley, N.J., King, B.J. *et al.* (2004) *Plant Physiol.*, **134**, 224–236.
22 Charron, J.B., Ouellet, F., Pelletier, M. *et al.* (2005) *Plant Physiol.*, **139**, 2017–2028.
23 Jain, M., Tyagi, A.K., and Khurana, J.P. (2006) *Genomics*, **88**, 360–371.
24 Silverstein, K.A., Moskal, W.A., Jr., Wu, H.C. *et al.* (2007) *Plant J.*, **51**, 262–280.
25 Boonburapong, B. and Buaboocha, T. (2007) *BMC Plant Biol.*, **7**, 4.
26 Jain, M., Nijhawan, A., Arora, R. *et al.* (2007) *Plant Physiol.*, **143**, 1467–1483.
27 Jain, M., Tyagi, A.K., and Khurana, J.P. (2008) *FEBS J.*, **275**, 2845–2861.
28 Kozian, D.H. and Kirschbaum, B.J. (1999) *Trends Biotechnol.*, **17**, 73–78.
29 Houston, N.L., Fan, C., Xiang, Q.-Y. *et al.* (2005) *Plant Physiol.*, **137**, 762–778.
30 Chardon, F. and Damerval, C. (2005) *J. Mol. Evol.*, **61**, 579–590.
31 Teixeira, F.K., Menezes-Benavente, L., Galvao, V.C. *et al.* (2006) *Planta*, **224**, 300–314.
32 Yang, X., Tuskan, G.A., and Cheng, M.Z. (2006) *Plant Physiol.*, **142**, 820–830.
33 Pareek, A., Singh, A., Kumar, M. *et al.* (2006) *Plant Physiol.*, **142**, 380–397.
34 Yuan, J.S., Yang, X., Lai, J. *et al.* (2007) *Funct. Integr. Geno.*, **7**, 1–16.

35 Kushwaha, H.R., Singh, A.K., Sopory, S.K. *et al.* (2009) *BMC Genomics*, **10**, 200.
36 Pareek, A., Singla-Pareek, S.L., Sopory, S.K., and Grover, A. (2007) *Analysis of Salt Stress-Related Transcriptome Fingerprints from Diverse Plant Species* (eds R.K. Varshney and R. Tuberosa), Springer, Dordrecht, pp. 267–287.
37 Rhee, S.Y., Beavis, W., Berardini, T.Z. *et al.* (2003) *Nucleic Acids Res.*, **31**, 224–228.
38 Kilian, J., Whitehead, D., Horak, J. *et al.* (2007) *Plant J.*, **50**, 347–363.
39 Weston, D.J., Gunter, L.E., Rogers, A., and Wullschlerger, S.D. (2008) *BMC Syst. Biol.*, **2**, 16.
40 Ma, S. and Bohnert, H.J. (2007) *Genome Biol.*, **8**, R49.
41 Li, Y., Zhu, Y., Liu, Y. *et al.* (2008) *Genomics*, **92**, 488–493.
42 Zeller, G., Henz, S.R., Widmer, C.K. *et al.* (2009) *Plant J.*, **58**, 1068–1082.
43 Adams, P., Nelson, D.E., Yamada, S. *et al.* (1998) *New Phytol.*, **138**, 171–190.
44 Golldack, D. and Dietz, K.J. (2001) *Plant Physiol.*, **125**, 1643–1654.
45 Kore-eda, S., Cushman, M., Akselrod, I. *et al.* (2004) *Gene*, **341**, 83–92.
46 Cushman, M.A., Bufford, D., Fredrickson, M. *et al.* (1999) *Plant Physiol.*, **120**, 145.
47 Cushman, J.C., Tillett, R.L., Wood, J.A. *et al.* (2008) *J. Exp. Bot.*, **59**, 1875–1894.
48 Van der Hoeven, R., Ronning, C., Giovannoni, J. *et al.* (2002) *Plant Cell*, **14**, 1441–1456.
49 Quackenbush, J., Cho, J., Lee, D. *et al.* (2001) *Nucleic Acids Res.*, **29**, 159–164.
50 Bernatzky, R. and Tanksley, S.D. (1986) *Mol. Gen. Genet.*, **203**, 8–14.
51 Fei, Z., Tang, X., Alba, R.M. *et al.* (2004) *Plant J.*, **40**, 47–59.
52 Katcrji, N., van Hoorn, J.W., Hanidy, A. *et al.* (2003) *Agr. Water Manage.*, **62**, 37–66.
53 Foolad, M.R. (2004) *Plant Cell Tissue Organ Cult.*, **76**, 101–119.
54 Sacher, R.E. and Staples, R.C. (1985) *Plant Physiol.*, **77**, 206–210.
55 Ouyang, B., Yang, T., Li, I.-I. *et al.* (2007) *J. Exp. Bot.*, **58**, 507–520.
56 Zhou, J., Wang, X., Jiao, Y. *et al.* (2007) *Plant Mol. Biol.*, **63**, 591–608.
57 Zhou, S., Sauve, R., Fish, T. *et al.* (2009) *J. Am. Soc. Hort. Sci.*, **134**, 289–294.
58 IRGSP (International Rice Genome Sequencing Project) (2005) *Nature*, **436**, 793–800.
59 Lutts, S., Kinet, J.M., and Bouharmont, J. (1995) *J. Exp. Bot.*, **46**, 1843–1852.
60 Xie, J.H., Zapata-Arias, F.J., Shen, M. *et al.* (2000) *Euphytica*, **116**, 105–110.
61 Senadheera, P., Singh, R.K., and Maathuis, F.J.M. (2009) *J. Exp. Bot.*, **60**, 2553–2563.
62 Walia, H., Wilson, C., Wahid, A. *et al.* (2006) *Funct. Integr. Genomics*, **6**, 143–156.
63 Chao, D.Y., Luo, Y.H., Shi, M. *et al.* (2005) *Cell Res.*, **15**, 796–810.
64 Zhou, S., Wei, S., Boone, B. *et al.* (2007) *Afr. J. Environ. Sci. Technol.*, **1**, 14–26.
65 Kumari, S., Sabharwal, V.P., Kushwaha, H.R. *et al.* (2009) *Funct. Integr. Genomics*, **9**, 109–123.
66 Kawasaki, S., Borchert, C., Deyholos, M. *et al.* (2001) *Plant Cell*, **13**, 889–905.
67 Ueda, A., Kathiresan, A., Bennet, J. *et al.* (2006) *Theor. Appl. Genet.*, **112**, 1286–1294.
68 Xu, Y., Skinner, D.J., Wu, H. *et al.* (2009) *Int. J. Plant Genomics*, **2009**, 957602.
69 Kollipara, K.P., Saab, I.N., Wych, R.D. *et al.* (2002) *Plant Physiol.*, **129**, 974–992.
70 Umezawa, T., Fujita, M., Fujita, Y. *et al.* (2006) *Curr. Opin. Biotechnol.*, **17**, 113–122.
71 Valliyodan, B. and Nguyen, H.T. (2006) *Curr. Opin. Plant Biol.*, **9**, 189–195.
72 Sreenivasulu, N., Sopory, S.K., and Kavi Kishor, P.B. (2007) *Gene*, **388**, 1–13.
73 Paterson, A.H., Bowers, J.E., Bruggmann, R. *et al.* (2009) *Nature*, **457**, 551–556.
74 Paterson, A.H. (2008) *Int. J. Plant Genomics*, **362**, 451.
75 Xu, W., Subudhi, P.K., Crasta, O.R. *et al.* (2000) *Genome*, **43**, 461–469.
76 Subudhi, P.K., Rosenow, D.T., and Nguyen, H.T. (2000) *Theor. Appl. Genet.*, **101**, 733–741.
77 Crasta, O.R., Xu, W., Rosenow, D.T. *et al.* (1999) *Mol. Gen. Genet.*, **262**, 579–588.
78 Haussmann, B.I.G., Mahalakshmi, V., Reddy, B.V.S. *et al.* (2002) *Theor. Appl. Genet.*, **106**, 133–142.

79 Buchanan, C.D., Lim, S., Salzman, R.A. *et al.* (2005) *Plant Mol. Biol.*, **58**, 699–720.

80 Sanchez-Barrena, M.J., Martinez-Ripoll, M., Zhum, J.K. *et al.* (2005) *J. Mol. Biol.*, **345**, 1253–1264.

81 Nakagami, H., Pitzschke, A., and Hirt, H. (2005) *Trends Plant Sci.*, **10**, 339–346.

82 Tanaka, Y., Sano, T., Tamaoki, M. *et al.* (2005) *Plant Physiol.*, **138**, 2337–2343.

83 Dinneny, J.R., Long, T.A., Wang, J.Y. *et al.* (2008) *Science*, **320**, 942–945.

84 Wang, M., Gu, D., Liu, T. *et al.* (2007) *Plant Mol. Biol.*, **65**, 733–746.

85 Kumar, V. (2000) *Mol. Biol. Rep.*, **27**, 45–49.

86 Brady, W.A., Kokoris, M.S., Fitzgibbon, M. *et al.* (1996) *J. Biol. Chem.*, **271**, 16734–16740.

87 Kumar, V., Spangenberg, O., and Konard, M. (2000) *Eur. J. Biochem.*, **267**, 606–615.

88 Liu, X.A. and Vance Baird, WmV. (2003) *Crop. Sci.*, **43**, 678–687.

89 Diédhiou, C.J., Popova, O.V., Dietz, K.J. *et al.* (2008) *BMC Plant Biol.*, **8**, 49.

90 Mukhopadhyay, A., Vij, S., and Tyagi, A.K. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 6309–6314.

91 Oono, Y., Seki, M., Nanjo, T. *et al.* (2003) *Plant J.*, **34**, 868–887.

92 Rabbani, M.A., Maruyama, K., Abe, H. *et al.* (2003) *Plant Physiol.*, **133**, 1755–1767.

93 Sottosanto, J.B., Gelli, A., and Blumwald, E. (2004) *Plant J.*, **40**, 752–771.

94 Davletova, S., Schlauch, K., Coutu, J. *et al.* (2005) *Trends Biotechnol.*, **23**, 547–552.

95 Cominelli, E., Galbiati, M., Vavasseur, A. *et al.* (2005) *Curr. Biol.*, **15**, 1196–1200.

96 Liang, Y.K., Dubos, C., Dodd, I.C. *et al.* (2005) *Curr. Biol.*, **15**, 1201–1206.

97 Hu, H., Dai, M., Yao, J. *et al.* (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 12987–12992.

98 Huang, X.Y., Chao, D.Y., Gao, J.P. *et al.* (2009) *Genes Dev.*, **23**, 1805–1817.

99 Hong, S.W., Jon, J.H., Kwak, J.M. *et al.* (1997) *Plant Physiol.*, **113**, 1203–1212.

100 Abe, H., Yamaguchi-Shinozaki, K., Urao, T. *et al.* (1997) *Plant Cell*, **9**, 1859–1868.

101 Yoo, J.H., Park, C.Y., Kim, J.C. *et al.* (2005) *J. Biol. Chem.*, **280**, 3697–3706.

102 Olsen, A.N., Ernst, H.A., Leggio, L.L. *et al.* (2005) *Trends Plant Sci.*, **10**, 79–87.

103 Zheng, X., Chen, B., Lu, G. *et al.* (2009) *Biochem. Biophys. Res. Commun.*, **379**, 985–989.

104 Sakuma, Y., Liu, Q., Dubouzet, J.G. *et al.* (2002) *Biochem. Biophys. Res. Commun.*, **90**, 998–1009.

105 Gutterson, N. and Reuber, T.L. (2004) *Curr. Opin. Plant Biol.*, **7**, 465–471.

106 Zhao, L., Hu, Y., Chong, K. *et al.* (2010) *Ann. Bot.*, **105**, 401–409.

107 Kizis, D. and Page's, M. (2002) *Plant J.*, **30**, 679–689.

108 Karaba, A., Dixit, S., Greco, R. *et al.* (2007) *Proc. Nat. Acad. Sci. USA*, **104**, 15270–15275.

109 Wang, Q., Guan, Y., Wu, Y. *et al.* (2008) *Plant Mol. Biol.*, **67**, 589–602.

110 Yin, Y.G., Kobayashi, Y., Sanuki, A. *et al.* (2010) *J. Exp. Bot.*, **61**, 563–574.

111 Jiang, Y. and Deyholos, M.K. (2006) *BMC Plant Biol.*, **6**, 25.

112 Moller, I.M. (2001) *Ann. Rev. Plant Physio. Plant Mol. Biol.*, **52**, 561–591.

113 Raes, J., Rohde, A., Christensen, J.H. *et al.* (2003) *Plant Physiol.*, **133**, 1051–1071.

114 Chao, D.Y., Luo, Y.H., Shi, M. *et al.* (2005) *Cell Res.*, **15**, 796–810.

115 Dure, L. (1993) *Plant J.*, **3**, 363–369.

116 Close, T. (1996) *Physiol. Plant*, **97**, 795–803.

117 Close, T. (1997) *Physiol. Plant*, **100**, 291–296.

118 Del Río, L.A., Sandalio, L.M., Corpas, F.J. *et al.* (2006) *Plant Physiol.*, **141**, 330–335.

119 Pitzschke, A. and Hirt, H. (2006) *Plant Physiol.*, **141**, 351–356.

120 Wang, W., Vinocur, B., Shoseyov, O. *et al.* (2004) *Trends Plant Sci.*, **9**, 244–252.

121 Sugino, M., Hibino, T., Tanaka, Y. *et al.* (1999) *Plant Sci.*, **146**, 81–88.

122 Zhu, J.K. (2001) *Trends Plant Sci.*, **6**, 66–67.

123 Wang, X., Liu, Z., and He, Y. (2008) *Plant Sig. Behav.*, **3**, 516–518.

124 Chen, X., Kanokporn, T., Zeng, Q. *et al.* (2002) *J. Exp. Bot.*, **53**, 225–232.

125 Kroemer, K., Reski, R., and Frank, W. (2004) *Plant Cell Rep.*, **22**, 864–870.

126 Lunde, C., Drew, D.P., Jacobs, A.K. *et al.* (2007) *Plant Physiol.*, **144**, 1786–1796.

127 Wang, X., Yang, P., Gao, Q. *et al.* (2008) *Planta*, **228**, 167–177.

128 Ratajczak, R. (2000) *Biochim. Biophys. Acta*, **1465**, 17–36.

129 Ratajczak, R. and Wilkins, T.A. (2000) *Energizing the Tonoplast* (eds D.G. Robinson and J.C. Rogers), Sheffield Academic Press Ltd., pp. 133–173.

130 Kavi Kishor, P.B., Hong, Z., Miao, G.-H. *et al.* (1995) *Plant Physiol.*, **25**, 1387–1394.

131 Kishitani, S., Takanami, T., Suzuki, M. *et al.* (2000) *Plant Cell Environ.*, **23**, 107–114.

132 Garg, A.K., Kim, J.K., Owens, T.G. *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 15898–15903.

133 Gollery, M., Harper, J., Cushman, J. *et al.* (2007) *Trends Plant Sci.*, **12**, 492–496.

134 Horan, K., Jang, C., Bailey-Serres, J. *et al.* (2008) *Plant Physiol.*, **147**, 41–57.

135 Gollery, M., Harper, J., Cushman, J. *et al.* (2006) *Genome Biol.*, **7**, 757.

136 Davletova, S., Rizhsky, L., Liang, H. *et al.* (2005) *Plant Cell*, **17**, 268–281.

137 Luhua, S., Ciftci-Yilmaz, S., Harper, J. *et al.* (2008) *Plant Physiol.*, **148**, 280–292.

138 Kawaura, K., Mochida, K., and Ogihara, Y. (2008) *Funct. Integr. Genomics*, **8**, 277–286.

139 Nemoto, Y. and Sasakuma, T. (2002) *Phytochemistry*, **61**, 129–133.

140 Barker, D.G., Bianchi, S., Blondon, F. *et al.* (1990) *Plant Mol. Biol. Rep.*, **8**, 40–49.

141 Trieu, A.T., Burleigh, S.H., Kardailsky, I.V. *et al.* (2000) *Plant J.*, **22**, 531–542.

142 Cook, D.R. (1999) *Curr. Opin. Plant Biol.*, **2**, 301–304.

143 Bell, C.J., Dixon, R.A., Farmer, A.D. *et al.* (2001) *Nucleic Acids Res.*, **29**, 114–117.

144 Nakano, T., Suzuki, K., Fujimura, T. *et al.* (2006) *Plant Physiol.*, **140**, 411–432.

145 Middleton, P.H., Jakab, J., Penmetsa, R.V. *et al.* (2007) *Plant Cell*, **19**, 1221–1234.

146 de Lorenzo, L., Merchan, F., Blanchet, S. *et al.* (2011) *Plant Physiol.*, **145**, 1521–1532.

147 Merchan, F., de Lorenzo, L., González-Rizzo, S. *et al.* (2007) *Plant J.*, **51**, 1–17.

148 Gruber, V., Blanchet, S., Diet, A. *et al.* (2009) *Mol. Genet. Genomics*, **281**, 55–66.

149 Henckel, K., Runte, K.J., Bekel, T. *et al.* (2009) *BMC Plant Biol.*, **11**, 9–19.

150 Takemura, T., Hanagata, N., Sugihara, K. *et al.* (2000) *Aquat. Bot.*, **68**, 15–28.

151 Banzai, T., Hershkovits, G., Katcoff, D.J. *et al.* (2002) *Plant Sci.*, **162**, 499–505.

152 Parida, A., Das, A.B., and Das, P. (2002) *J. Plant Biol.*, **45**, 28–36.

153 Muthukumarasamy, M., Gupta, S.D., and Pannerselvam, R. (2000) *Biol. Plant.*, **43**, 317–320.

154 Wonga, Y.-Y., Hoa, C.-L., Nguyena, P.D. *et al.* (2007) *Aquat. Bot.*, **86**, 117–122.

155 Tada, Y. and Kashimura, T. (2009) *Plant Cell Physiol.*, **50**, 439–446.

156 Seki, M., Narusaka, M., Abe, H. *et al.* (2001) *Plant Cell*, **13**, 61–72.

157 Ozturk, Z.N., Talame, V., Deyhoyos, M. *et al.* (2002) *Plant Mol. Biol.*, **48**, 551–573.

158 Ueda, A., Kathiresan, A., Inada, M. *et al.* (2004) *J. Exp. Bot.*, **55**, 2213–2218.

159 Ueda, W.S., Nakamura, T., and Takabe, T. (2002) *J. Plant Res.*, **115**, 119–130.

160 Martinez, C.A., Maestri, M., and Lani, E.G. (1996) *Plant Sci.*, **116**, 177–184.

161 Benevides, M.P., Marconi, L.P., Gallego, S.M. *et al.* (2000) *Aust. J. Plant Physiol.*, **27**, 273–278.

162 Rahnama, H. and Ebrahimzadeh, H. (2004) *Acta Physiol. Plant*, **26**, 263–270.

163 Tang, L., Kwon, S.Y., Kim, S.H. *et al.* (2006) *Plant Cell Rep.*, **25**, 1380–1386.

164 Rensink, W.A., Iobst, S., and Hart, A. (2005) *Funct. Integr. Geno.*, **5**, 201–207.

165 Sayari, A.H., Bouzid, R.G., Bidani, A. *et al.* (2005) *Plant Sci.*, **169**, 746–752.

166 Aghaei, K., Ehsanpour, A.A., and Komatsu, S. (2008) *J. Proteome Res.*, **7**, 4858–4868.

167 Maas, E.V. and Hoffman, G.J. (1977) *J. Irrig. Drain. Div. ASCE*, **103**, 115–134.

168 Hawker, J.S. and Walker, R.R. (1978) *Am. J. Enol. Vitic.*, **29**, 172–176.

169 Shani, U., Waisel, Y., Eshel, A. *et al.* (1993) *New Phytol.*, **124**, 695–701.

170 Walker, R.R., Blackmore, D.H., Clingeleffer, P.R. *et al.* (2002) *Aust. J. Grape Wine Res.*, **8**, 3–14.

171 Cramer, G.R., Ergül, A., Grimplet, J. *et al.* (2007) *Funct. Integr. Genomics*, **7**, 111–134.

172 Doddapaneni, H., Lin, H., Walker, M.A. *et al.* (2008) *BMC Plant Biol.*, **8**, 23.

173 Bressan, R.A., Zhang, C., and Zhang, H. (2001) *Plant Physiol.*, **127**, 1354–1360.

174 Inan, G., Zhang, Q., Li, P. *et al.* (2004) *Plant Physiol.*, **135**, 1718–1737.

175 Gong, Q.Q., Li, P.H., Ma, S.S. *et al.* (2005) *Plant J.*, **44**, 826–839.

176 Amtmann, A., Bohnert, H.J., and Bressan, R.A. (2005) *Plant Physiol.*, **138**, 127–130.

177 Taji, T., Seki, M., Satou, M. *et al.* (2004) *Plant Physiol.*, **135**, 1697–1709.

178 Kant, S., Kant, P., Raveh, E. *et al.* (2006) *Plant Cell Env.*, **29**, 1220–1234.

179 Ghars, M.A., Parre, E., Debez, A. *et al.* (2008) *J. Plant Physiol.*, **165**, 588–599.

180 Wong, C.E., Li, Y., Labbe, A. *et al.* (2006) *Plant Physiol.*, **140**, 1437–1450.

181 Oh, D.-H., Gong, Q.Q., Ulanov, A. *et al.* (2007) *J. Integr. Plant Biol.*, **49**, 1484–1496.

182 Oh, D.-H., Leidi, E., Zhang, Q. *et al.* (2009) *Plant Physiol.*, **151**, 210–222.

183 Liu, N., Chen, A.P., Zhong, N.Q. *et al.* (2007) *Physiol. Plant*, **129**, 671–678.

184 Ni, W.S., Lei, Z.Y., Chen, X. *et al.* (2007) *J. Integr. Plant Biol.*, **49**, 1313–1319.

185 Sengupta, S. and Majumder, A.L. (2010) *Plant Cell Env.*, **33**, 526–542.

186 Sengupta, S. and Majumder, A.L. (2009) *Planta*, **229**, 911–929.

187 Singla-Pareek, S.L., Reddy, M.K., and Sopory, S.K. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 14672–14677.

188 Singla-Pareek, S.L., Yadav, S.K., Pareek, A., Reddy, M.K., and Sopory, S.K. (2006) *Plant Physiol.*, **140**, 613–623.

2
Abiotic Stress Tolerance in Plants: An Industry Perspective

Shoba Sivasankar, Robert W. Williams, and Thomas W. Greene

After insect resistance and herbicide resistance, agronomic traits that define the extent and stability of economic yield are becoming recognized as the next-generation plant biotechnology traits. The stability of yield is determined by the tolerance of the plant to abiotic stresses, such as drought, cold, salt, and heat. Considerable research in the public and private sectors is devoted to the development of abiotic stress tolerance in plants, a good component of this being at the level of high-throughput gene discovery and gene evaluation. A functional relationship to stress tolerance has been demonstrated in model plant species for roughly 200 genes over the past 30 years. The challenge is to translate this functional efficacy to field performance, as demonstrated in reduced yield loss under stress. In addition, the extent of yield stability conferred by individual genes must be enhanced to the level at which it becomes commercially viable. While the complexity and multigene nature of abiotic stress tolerance make it a challenging undertaking, early indications of success are evident from research in both the plant biotechnology industry and the academia. In this chapter, we describe the various approaches to gene discovery used for abiotic stress tolerance, and discuss the challenges of high-throughput phenomics under controlled environment conditions. We conclude with a brief overview of the recent breakthroughs in abiotic stress research in the plant biotechnology industry.

2.1
Introduction

The postgenomics era, with its influx of enhanced technology in genome-scale transcript profiling, computational biology, metabolomics, and phenomics, has enabled continuously improving capabilities to explore functional plant biology. This makes it possible to address hitherto unexplored aspects of fundamental and applied crop research at high levels of throughput, though not without challenges. Consequently, the competitive landscape in the seed and plant biotechnology

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industry is constantly changing, with existing players enhancing their research capabilities through mergers, acquisitions, and global extension of research facilities, and newer players rapidly filling emerging technological niches. First-mover advantage in existing and emerging seed and seed-related businesses is determined, as always, by the ability to deliver enhanced, stable yields under both optimal and challenging growth environments.

Agronomic traits that define the extent and stability of economic yield are rapidly becoming recognized as the next-generation plant biotechnology traits of commercial significance, following insect resistance and herbicide resistance. The extent of yield produced by a given crop species is determined both by its intrinsic yield potential and its capacity to perform under adverse growing conditions imposed both by abiotic and biotic stresses and by nutrient limitation. Narrowing the yield gap that exists between yield potential and realized yield is the subject of intense breeding and biotechnology research in commercial crop species. Development of crop plants that deliver enhanced and stable yields under changing climatic conditions, with the occurrence of multiple abiotic stresses during a single growing season, is a challenging undertaking. It requires (1) a mechanistic understanding of the response, acclimation and tolerance to individual stress conditions, and to naturally occurring combinations of multiple stresses; (2) the identification of critical physiological processes, biochemical pathways, allelic variants, and protein conformations associated with stress response and stress tolerance; (3) careful attention to precision phenotyping, growing conditions, and field heterogeneity in the testing process; and (4) the integration of these with transgenic efforts, QTL mapping, molecular breeding, and modeling [1–6].

Abiotic stresses such as drought, cold, and salt are of wide occurrence and have significant impact on crop productivity, and as such, tolerance to these stresses is a critical condition for yield stability. Tolerance to abiotic stress is complex and involves multiple component traits, physiological responses, and biochemical pathways. This complexity necessitates continued emphasis on efforts to identify and understand the morphophysiological, biochemical, and molecular components associated with tolerance, and to associate the contribution of these with yield stability and final yield. Whole-plant physiology combined with traditional breeding over the last several years has identified several component traits that contribute to yield stability under stress, especially with relevance to drought tolerance. Today, the establishment and continuous enhancement of high-throughput facilities including genome-scale transcript profiling, metabolomics, and phenomics capabilities are helping to achieve economies of both scale and scope that inform efforts toward crop improvement. Challenges continue to remain in the determination of associations between molecular events and whole-plant-level morphophysiological traits relevant to abiotic stress tolerance, and the utilization of such understanding for the development of commercial seed products.

Although abiotic stress tolerance is a typical quantitative trait, there are examples of single genes such as those controlling flowering time, plant height, ear type, and osmotic adjustment playing important roles in plant adaptation to stress, as analyzed

in relation to the impact of quantitative trait loci (QTL) in breeding for tolerance [7, 8]. However, the fact that numerous genes are involved in the expression of polygenic traits means that the individual genes generally have small effects at the phenotype level, and thus may need to be combined effectively in order to obtain a significant commercial impact. Breeding to manipulate more than one quantitative trait locus contributing to relevant traits in abiotic stress tolerance can help increase the impact on the phenotype. Biotechnology can enhance existing natural variation for the trait, and can facilitate the delivery of genetic variation not present in the crop species to be modified.

At the molecular level, the complex nature of abiotic stress tolerance can be evaluated by genome-wide evaluation of transcripts to identify genes with an association with the trait of interest, especially important when trait-relevant functionality of less than 1% of the genome has been established at the present time. Genome-wide targeted queries for assessment of trait-relevant functionality of transcripts are possible through forward genetics approaches such as the phenotypic evaluation of populations of activation-tagged, transposon-tagged, or T-DNA insertion mutants. Alongside of this, more defined forward genetics approaches and reverse genetics approaches in high-throughput mode are prevalent in the plant biotechnology industry. These include evaluation of functional gene classes such as transcription factors, gene families where more than one member has proven to be involved in the response or tolerance to abiotic stress, genes and gene networks predicted through computational biology, and stress-related transcripts from extremophile plant or bacterial species. In addition, comparative genomics, global profiling, knowledge-based gene discovery and directed evolution all play a role in identifying and applying molecular information to trait improvement.

Functional analysis of genes through phenomics and metabolomics approaches helps to unravel the complexity of the trait, to understand similarities and differences across plant species or within species, and to better define stress-adaptive metabolic pathways and regulatory gene networks. Integrated information from these functional analyses platforms can be structured into testable hypothesis with the aim to improve abiotic stress tolerance in commercial crop species, and for the continuous improvement of trait-specific screening under controlled environments and in the field. Leveraging information from high-throughput phenotypic screens of model systems to crop improvement, and the relationship between model system-based gene-to-phenotype confirmation and gene(s)-to-yield applicability, continues to be a subject of intense discussion.

In this chapter, we discuss recent trends in abiotic stress research in the plant biotechnology industry, emphasizing drought. Our focus is upon gene discovery through a variety of approaches, and upon functional analysis and evaluation of the plant phenotype conferred by selected genes through the use of phenomics approaches. We describe important challenges and breakthroughs in the development and deployment of stress-tolerant commercial seed products in the private sector.

2.2
Gene Discovery and Genomics in the Plant Biotechnology Industry

Research in the plant biotechnology industry aims to identify genes or "leads" that are capable of conferring a definitive phenotype in relation to the trait of interest, transfer of these leads into commercial crop species for evaluation of the resultant transgenic plants in the green house and field environments, and optimization of transgenic performance as necessary to deliver desirable agronomic performance in the farmer's field. With the completion of the DNA sequencing of the *Arabidopsis* genome in 2000, the subsequent DNA sequencing of the rice and sorghum genomes and, most recently, the release of draft genomes of wheat and maize through the last decade, the principal challenge to plant biologists has become the assignment of functionality to the sequenced genes and the association of genes with definitive phenotypes. In certain instances, the plant biotechnology industry has contributed to sequence information of crop species to publicly released genome information [9]. Sequence information of complete genomes of model plant species or commercial crop species has significantly enhanced the ability to identify genes associated with traits of interest in both the public and the private sectors.

In the broadest sense, there are two approaches to discover genes that have the potential to impart abiotic stress tolerance, or any trait, to plants, and these are forward and reverse genetics. Forward genetics identifies the phenotype of interest from screening populations and follows up with the cloning and identification of the gene(s) sufficient to confer the phenotype. Reverse genetics, on the other hand, works from the gene to the phenotype. It starts with a gene hypothesized to be involved in a specific phenotype and attempts to identify an alteration in the phenotype through perturbed expression of the gene. The distinction between forward and reverse genetics approaches is not necessarily absolute, and whole gene families or functional classes with potential to impact the trait of interest can be assessed for their effect on the phenotype.

In the following sections, we describe the methods of gene discovery and gene modification for trait improvement most commonly used in the plant biotechnology industry, including forward genetics, evaluation of gene families or functional gene classes, knowledge-based gene discovery, directed evolution, global profiling, comparative genomics, and computational biology.

2.2.1
Forward Genetic Screens Using Model Species

Forward genetics helps to associate a phenotype with a gene with no *a priori* assumptions about gene function or annotation. The widest adaptation of this approach involves the query of entire genomes through the use of mutant populations in which the expression of a good proportion of genes in the genome has been altered through the insertion of T-DNA, transposon, or activation tags [10–13]. The use of chemical or physical treatments such as ethyl methane sulfonate (EMS) or fast neutrons to generate mutant populations with disrupted gene expression is still

prevalent, though to a lesser extent. The process of identifying trait-relevant genes through forward genetics approaches involves a series of steps. First, tagged or insertion mutant populations are generated in the model species of choice, predominantly, *Arabidopsis thaliana*. Next, high-throughput phenotyping is executed on these populations to identify positive or negative effects on chosen secondary or component traits associated with the trait of interest. With this phenotyping process, mutants that produce definitive qualitative or quantitative evidence for altering chosen secondary traits of focus are identified. This is followed by cloning the gene whose activation or disruption produced the phenotype of interest and then validating its effect by retransformation into the same model species to recapitulate the mutant phenotype, using constructs designed to produce the desired expression. The last step involves the introduction of the confirmed or validated gene into the crop species of choice, and evaluation of the resultant transgenic plants in the greenhouse and/or the field.

The development of tagged populations in model plant species and their screening for the identification of genes associated with specific traits, including abiotic stress tolerance, is a practice as prevalent in the plant biotechnology industry as in academic research. One of the early examples of forward genetics approaches for response to multiple abiotic stresses attempted to identify genes responding to low temperature, drought, salinity, and the phytohormone, abscisic acid (ABA), utilizing bioluminescence of the luciferase reporter gene expressed from the dehydration-responsive promoter *RD(RESPONSIVE TO DEHYDRATION) 29A* [14]. This group screened an EMS-mutagenized *Arabidopsis* population homozygous for the transgenic construct, *RD29A::LUC (LUCIFERASE)* and identified several genes responsive to dehydration, salinity, low temperature, and ABA. Of these, *LOS5 (LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES)* is under evaluation by FuturaGene Plc. (recently acquired by SuzanoTrading Ltd) for crop improvement, as part of FuturaGene's research portfolio that focuses on yield, abiotic stress tolerance, forestry, and biofuels (http://www.futuragene.com/06annual.pdf).

Dupont Agricultural Biotechnology and Pioneer Hi-Bred International, Inc. use activation-tagged populations of *A. thaliana* to screen for abiotic stress tolerance, keying in on critical secondary or component traits of relevance. The Monsanto Company has also reported the use of activation T-DNA tagging in *Arabidopsis* to obtain gain-of-function mutants related to critical traits [15]. *A. thaliana*, by virtue of its fully sequenced small genome, small plant size, and short growing period, need not necessarily be the only plant species utilized in forward genetics approaches to identify genes with the potential to confer abiotic stress tolerance. Both model species and commercial crop species with fully elucidated genomes can serve as targets for the development of screening populations. Ideally, in addition to having completely sequenced genome information and lending itself to easy cultivation in a high-throughput mode, these species should possess at least some characteristics of extremophiles, specifically in relation to drought, salt, or cold stress.

Thellungiella salsuginea (also known as *T. halophila*), a close relative of *A. thaliana*, is one such model species that is being explored for abiotic stress tolerance and nitrogen use efficiency [16–18]. Similar to *Arabidopsis*, it has a short lifecycle, small genome,

copious seed production, and is easily amenable to the floral-dipping method of plant transformation. Unlike *Arabidopsis*, however, it has the additional advantage of growing well under extreme conditions of cold, salt, and drought stress, and under nitrogen-limited conditions. *Thellungiella* genes exhibit high sequence identity (approximately 90% at the cDNA level) with *Arabidopsis* genes, and its extremophile characteristics result mostly from regulation of gene expression and to a lesser extent from a limited number of unique gene [19]. While full-genome sequencing is pending for this species, cDNA expression libraries have been transformed into *Arabidopsis*, and the resulting transgenic populations have been evaluated for abiotic stress responses [19, 20].

Physcomitrella patens, a moss highly tolerant to drought, salt, and osmotic stress, can serve as another excellent model species for forward genetics approaches in abiotic stress tolerance [21, 22]. A draft genome for this haploid is available, and the species is amenable to forward genetics approaches with faster turnaround rates than *A. thaliana* [23, 24]. Yet another model plant species, *Brachypodium distachyon*, can also render itself to the development of screening populations and can serve as a source of unique genes for trait improvement of commercial crop species [25]. *B. distachyon* is a wild grass related to small grain cereals, and is the first member of the Pooideae subfamily of grasses to be sequenced. Though its performance under abiotic stress conditions is not clearly described, its high-quality genome sequence, small genome, ease of cultivation and transformation, small size, and rapid life cycle all make it a good model species for gene discovery and phenomics. The extent of use of screening populations involving *Thellungiella*, *Physcomitrella*, *or Brachypodium* or model crop species for identification of genes of relevance to abiotic stress tolerance in the plant biotechnology industry is not known.

2.2.2
Functional Gene Classes and Families

A very good example of the evaluation of functional gene classes and gene families for the associated phenotype, including the ability to confer abiotic stress tolerance to plants, is the evaluation of the *A. thaliana* transcription factors by Mendel Biotechnology, Inc. (http://www.mendelbio.com/technology/index.php). Several research groups in both the public and the private sectors have used systematic functional genomics strategies to identify transcription factors with commercial potential. The *Arabidopsis* genome encodes over 1500 transcription factors, which account for roughly 5% of its genome, and about 45% of these transcription factors are unique to plants [26, 27]. Mendel Biotechnology was founded in 1997, with a focus on understanding the function of the large class of transcription factor genes in *A. thaliana*, based on the premise that these are upstream regulators capable of regulating and coordinating the expression of multiple downstream effectors that together confer specific plant characteristics.

The unique characteristics and modes of action of transcription factors make an overexpression strategy more attractive for assessment of gene function than does a knockout strategy [28]. As described on the Mendel Biotechnology web page

(http://www.mendelbio.com/aboutus/history.php), company scientists systematically analyzed the function of all *Arabidopsis* transcription factors, by overexpression in *A. thaliana*, and discovered individual transcription factors that control complex traits such as freezing tolerance, drought tolerance, intrinsic growth rate, photosynthetic output, plant form, disease resistance, water use efficiency, nitrogen use efficiency, and numerous other important processes. A well-studied class of transcription factors at Mendel is the AP2 transcription factor class, *C-BOX BINDING FACTOR(CBF)/DEHYDRATION-RESPONSIVE ELEMENT BINDING(DREB)* genes that are being evaluated for commercial development [29]. The *CBF* genes have been successfully used to engineer abiotic stress tolerance in a number of plant species [30]. According to public information (http://www.arborgen.us/index.php/products/product-pipeline/freeze-tolerant-eucalyptus), a commercial application for the *CBF* genes is closest to reality at Arborgen Inc., in the case of eucalyptus trees where *CBF* has been shown to contribute to freezing tolerance. The implication of another class of transcription factors, the *NF-Y* family, in drought tolerance in maize at Monsanto as described later, is a collaborative effort between Mendel and Monsanto [31]. For a commercial product, it is important to note that transcription factor technologies may require optimization mostly through the use of stress-inducible promoters, either to overcome pleiotropic effects resulting from constitutive overexpression or to enhance the desired trait to the level at which it is commercially viable. With the advent of "systems biology" approaches in the postgenomics era, it is possible to assemble all genes in the genome into transcription networks or protein interaction networks underpinning major biological processes. This enables researchers to investigate potential intervention points and gene combinations for coregulated expression to achieve abiotic stress tolerance and yield stability [32].

Other than transcription factors, a class of genes that has been evaluated through reverse genetics is involved in cell cycle regulation, including genes encoding the *KIP-RELATED PROTEINS (KRPs)* that are negative regulators of cell cycle [33]. Regulation of cell cycle is crucial to plant growth and development, and similar to the case of transcription factors mentioned above, plants have several unique genes involved in the process, though the cell cycle process in plants shares basic mechanisms with other eukaryotes [34]. The *KRP* genes are under advanced studies for commercial application both in canola and in camelina, an alternative oilseed energy crop, in the hands of Sustainable Oils, Inc. and one of its parent companies, Targeted Growth, Inc. (http://www.susoils.com/camelina/researchadv_resdev.php).

Genes involved in cell cycle, along with other classes of genes, have been subject to reverse genetics evaluation at Crop Design, founded in 1998 as a spin-off of the research work from Dirk Inze's group at the Flanders Inter-University Institute of Biotechnology, Ghent, Belgium. Crop Design's trait discovery and development program focuses on enhancing grain yield in corn and rice, and utilizes its TraitMill™ platform for applied genomics and phenomics. As indicated on Crop Design's web page (http://www.cropdesign.com/general.php), the company has discovered a range of proprietary leads through TraitMill™ for application in the areas of yield enhancement, drought tolerance, and improved nutrient use efficiency.

The TraitMill™ platform utilizes proprietary bioinformatics tools, high-throughput gene engineering systems, efficient methods for plant transformation, and automated high-throughput high-resolution phenotypic evaluation of transgene performance in rice (http://www.cropdesign.com/tech_traitmill.php).

2.2.3 Knowledge-Based Gene Discovery

In this approach, specific pathways known to be associated with a physiological trait or biochemical pathway that is triggered in response to a given abiotic stress is chosen for identifying key genes that can be targets for biotechnology. Examples are *POLY (ADP-RIBOSE) POLYMERASE (PARP)* and *POLY(ADP-RIBOSE) GLYCOHYDROLASE (PARG)* genes from Bayer CropScience (http://www.research.bayer.com/edition_16/16_Biotechnology.pdfx), and protein farnesyltransferase that forms the Yield Protection Technology® from Performance Plants, Inc. (http://performanceplants.com/technology-products/technology-solutions/yield-protection-technology). Stress-induced activation of *PARP* is the main cause of energy depletion in animals. Plants have two *PARP* genes, *PARP1* and *PARP2*, both of which are activated by DNA damage under stress, leading to energy depletion through NAD ($^+$) breakdown and ATP consumption. Inhibition of the activity or gene expression of these proteins, by chemical inhibitors or transgenic downregulation, contributes to tolerance to a broad range of abiotic stresses such as high light, drought, and heat. In this example, the focus was on improving energy efficiency under stress conditions, an aspect that is still not explored to the extent it deserves in the area of plant abiotic stress research [35]. Follow-up studies have shown that in addition to enhanced energy use efficiency, transgenic plants with downregulated *PARP* had altered levels of the hormone ABA that resulted in the induction of a wide range of defense-related genes [36]. Most recently, the *SNF1-RELATED PROTEIN KINASE, SnRK 2.6*, has been implicated in metabolic homeostasis and energy balance in *A. thaliana*, adding to earlier reports of similar roles for members of the *SnRK1* subfamily in plants [37]. This role of *SnRK2.6* is quite apart from its role as a positive regulator in ABA signaling, and its involvement in regulating ABA-mediated stomatal aperture.

Development of drought tolerance through manipulation of the response to ABA has been attempted across a variety of studies, and Performance Plants, Inc. has shown the involvement of the gene encoding the beta subunit of *Arabidopsis* farnesyltransferase, *ENHANCED RESPONSE TO ABA (ERA1)* in the regulation of ABA sensing and drought tolerance [38]. The *era1* mutant was identified from a fast neutron- and T-DNA-mutagenized *Arabidopsis* population for supersensitivity to ABA [39]. Downregulation of either the alpha or the beta subunit of farnesyltransferase enhanced the plant's response to ABA and drought tolerance [39]. This work specifically demonstrated that antisense constructs for the *Brassica napus ERA1* expressed from the dehydration stress-responsive promoter, *RD29A*, can produce transgenic plants with enhanced tolerance to moderate drought and significantly higher seed yield than control plants across 3 consecutive years of field test. Under drought conditions, these transgenic plants showed significant reduction in stomatal

conductance and transpiration, and were more resistant to drought-induced seed abortion during flowering relative to nontransgenic control plants. When grown under conditions of adequate moisture in the field, the yield of transgenic plants was the same as that of nontransgenic control plants.

2.2.4 Directed Molecular Evolution

Directed molecular evolution through multigene shuffling is a powerful technique to modify protein characteristics including improvement in specific activity or enzyme kinetics, formation of novel substrate specificities or novel products, and optimal performance in specific environments [40]. It has been successfully used in the generation of novel carotenoids, enhanced herbicide detoxification, and improvement of insect resistance genes. Maxygen Incorporated, California, USA, founded in 1997, uses its proprietary MolecularBreeding™ shuffling technology for the development of superior proteins, and its subsidiary, Verdia, has been a part of Dupont Agricultural Biotechnology since 2004, focusing on plant biotechnology. In the area of abiotic stress tolerance, Kurek *et al.* at Dupont have reported the generation of transgenic *Arabidopsis* with wild-type *RUBISCO ACTIVASE (RCA)* or *RCA* that had been shuffled for enhanced thermostability [41]. They observed enhanced photosynthesis, plant growth, and seed production under heat stress in the transgenic plants harboring the shuffled protein relative to the control plants transformed with the wild-type protein.

2.2.5 Global Profiling

With the elucidation of the complete genomes of both model plant species and commercial crop plants, and the continuous improvement of genome-scale transcript profiling methods, global transcript profiling is used widely in both the public and the private sectors to determine changes in gene expression under a variety of perturbations, including exposure to abiotic stresses. While the information from transcript profiling is valuable for understanding stress response at the gene expression level, it also serves as a source of genes for transgenic modulation or for marker-assisted breeding, when combined with other relevant data. Reported examples of the work in the private sector in transcriptomics includes that of Zinselmeier *et al.*, as well as Kollipara *et al.* at Pioneer-DuPont, and that of Kreps *et al.* at Syngenta [42–44]. Zinselmeier *et al.* focused on reproductive development in maize under drought stress, a developmental window critical for yield stability under drought, to conduct gene expression profiling using microarrays of maize expressed sequence tags [44]. While confirming the significance of the starch biosynthetic pathway in the regulation of stress responses, they also identified new pathways and genes in maize associated with drought stress. Again, at Pioneer-DuPont, Kollipara *et al.* reported a parallel transcriptomic and proteomic study across recombinant inbred lines of maize derived from B73 X Mo17 that showed differential behavior for

cold germination and seed desiccation tolerance [42]. This group identified a variety of genes and proteins that responded differentially to the two stresses, which could be classed into various functional groups such as carbohydrate and amino acid metabolism, ion transporters, stress and defense response, polyamine metabolism, chaperonins, cytoskeleton associated, and so on.

At Syngenta, researchers used microarray-based expression profiling of *Arabidopsis* responding to salt, osmotic, and cold stresses to identify a combination of over 2000 sequences that showed differential response across the three stresses [43]. Of all these differentially expressed sequences, the one that had the largest induction across all three stresses was identified to be *At5g52310* (*COLD RESPONSIVE 78 or COR78*). The above reports are from a period when transcript profiling was primarily done using microarrays with expressed sequence tags, and did not cover the entire transcriptome. However, EST- and oligonucleotide-based microarrays are likely to become obsolete in the near future with affordable and fast gene-expression analysis by cDNA sequencing.

Analysis of all available expression data for a given species can help identify *cis*-elements involved in the regulation of gene expression, when combined with genome sequence and transcript information, as has been reported by researchers at Ceres, Inc. [45]. Across the *Arabidopsis* genome, this group identified motifs associated with drought, heat, and cold stress, along with six other traits or tissue types. They were also able to validate the association of these *cis*-elements with known responses or tissue types, as in the case of the ABA responsive element, CACGT, which is known to be associated with drought, and was also strongly expressed in the drought data set the authors used.

Though significant research has been published in the public sector on global proteomic analysis in response to abiotic stress [46, 47], reports from the private sector are limited, one of those being the results from the work of Kollipara *et al.* mentioned above. Similar is the case with metabolomics, though this is an area receiving increasing interest in both the private and the public sectors due to enhanced capabilities leading to deciphering fundamental aspects of plant cell systems and their application to trait discovery [48, 49]. Metanomics GmbH, Berlin, Germany, with its focus on metabolic functional genomics, has developed large *Arabidopsis* populations with overexpression of an entire prokaryotic and an entire eukaryotic genome (http://www.metanomics.de/seiten/frameset.html). This population is being explored for commercially important metabolite changes, and has led to the identification and validation of several lead genes associated with tolerance to environmental stresses, as well as traits.

At Monsanto, Harrigan *et al.* conducted metabolite analysis of the grain of seven maize hybrids subjected to three separate moisture regimes during growth, namely, well-watered conditions, moisture stress during vegetative growth, and moisture stress during grain filling [50]. Their results showed that the magnitude of the mean differences between well-watered and drought-stressed samples were small, and statistically significant drought-induced differences were observable in at least four of the seven hybrids for only glutamine and proline of the various analytes measured.

2.2.6 Comparative Genomics

Owing to naturally occurring variations in the levels of tolerance and adaptation to abiotic stresses both between plant species and within species (as between wild progenitors and cultivated varieties), a comparative evaluation of the expression of these responses at the molecular level is critical to the identification of pathways and genes that are of significance to the tolerance response. In addition to pinpointing critical pathways and genes of interest, comparative genomics allows the evaluation of differences in the levels and timing of gene expression between tolerant and susceptible species or varieties, which contribute to their specific response to the stress. Furthermore, comparative genomics also provides an avenue for drilling into allelic differences in the regulatory regions of genes that lead to gene expression differences between closely related species or between varieties within the same species. Comparative genomics can be done at a global scale covering the entire genome or can be focused on specific classes of genes known to be associated with the trait of interest. Most published reports in this area are confined to the published genome sequences of *Arabidopsis* and rice.

Examples of focused comparative genomics of gene classes with relevance to abiotic stress research are the analysis of the *CCCH ZINC FINGER* and *PROTEIN PHOSPHATASE 2C (PP2C)* families in *Arabidopsis* and rice in the public sector [51, 52]. Genes encoding the former are known to play important roles in RNA processing as RNA binding proteins in animals. A role for these proteins in plants is only just emerging and the induction of their expression upon exposure to stress conditions implicates an association with the response to abiotic and biotic stresses. The *PP2C* family is associated with negative modulation of kinases involved in environmental stress responses and developmental processes, and their comparative evaluation across the genomes of *Arabidopsis* and rice has identified both similarities and differences in their response to abiotic stress and ABA. Expanding comparative genomics analysis to other stress-related gene families, to species beyond *Arabidopsis* and rice, and to varieties within species will be useful in understanding the tolerance response in terms of expression modulation. This is in addition to identifying novel divergent sequences that can serve as candidates for biotechnological interventions.

In addition to the above, the effect of transgenic perturbations or quantitative trait loci can be evaluated by comparative genomic analysis of the wild-type genome and the perturbed genome. In a collaborative effort between researchers at Dupont Agricultural Biotechnology and the Rockefeller University, the genome-wide expression profile of wild-type *Arabidopsis* was compared with that of the *ABA-INSENSITIVE* mutant, *abi1-1*, using massively parallel signature sequencing [53]. This work established that the regulation of gene expression of a majority of ABA-responsive genes was abolished in the mutant. On the basis of the response of wild-type *Arabidopsis* to ABA, the group identified more than a 1000 genes responding to ABA, including novel ABA-responsive pathways and genes.

A similar study reported by Syngenta and collaborators evaluated the expression profiles of parental and double-haploid rice accessions in an attempt to determine

general changes in gene expression under dehydration stress, differences in gene expression between parental lines with high or low osmotic adjustment, and differences in gene expression of transgressive segregants from these parental lines, with opportunity to identify candidate genes based on previously established drought tolerance QTL [54]. They identified 69 genes differentially upregulated in lines with high osmotic adjustment, 9 of which were not induced to any extent in lines with low osmotic adjustment. The latter included *SUCROSE SYNTHASE*, a pore protein, a *HEAT SHOCK PROTEIN*, and a *LATE EMBRYOGENESIS ABUNDANT* protein or *LEA*. Furthermore, this effort helped identify candidates associated with the drought tolerance QTL, which included a *snRNP AUXILIARY FACTOR*, a *LEA* protein, a *PROTEIN PHOSPHATASE 2C*, and a *SAR1* homologue.

2.2.7
Computational Biology

Advanced computational tools and specific algorithms can be applied to collections of large amounts of proprietary and/or public genomic information to enable the identification of key genes that could be critical targets for modification to alter traits of interest. Evogene Ltd, Rehovot, Israel, utilizes its proprietary "ATHLETE" platform (Agro Traits Harvest LEeads Technology) for such *en silico* gene identification and evaluates the identified genes in model plant systems prior to transfer and evaluation in target crop species (http://www.evogene.com/technology.asp?tid=7). Evogene also utilizes comparative genomics and utilizes evolutionary information across 70 plant species and approximately 8 million expressed sequence tags to facilitate gene identification. Researchers at Evogene, in association with the Hebrew University of Jerusalem, used computational mining of the large multifunctional gene family of tomato aquaporins on the basis of gene induction in response to abiotic stresses to identify one specific aquaporin, *SOLANUM LYCOPERSICUM TONOPLAST INTEGRAL PROTEIN 2;2* or *SlTIP2;2* [55]. Transgenic plants overexpressing this gene showed increased cell osmotic water permeability and enhanced whole-plant transpiration rates. When subjected to commercial greenhouse trials, the transgenic tomato plants showed increased fruit yield, harvest index, and plant biomass under both well-watered and drought-stressed conditions over 3 consecutive years.

2.3
High-Throughput Phenotyping and Phenomics

Following the trend of "omics" terminologies at the molecular level, phenomics is a term that is coming into increasing use to describe high-throughput precision phenotyping that enables evaluation of the physiological and morphological aspects of the plant under controlled environments or in the field. Phenomics methodologies are used to identify genes associated with the trait of interest by helping to establish functional relationship between genetics and the associated phenotype. They are also used to characterize plant performance under controlled environments and in the

field. In developing yield stability under abiotic stress conditions such as drought and cold, it is important to recognize that the selection can be targeted for escape or for tolerance. Escape responses are generally associated with a shorter growth duration that allows the plant to avoid periods of natural occurrence of the stress. This is possible in geographical locations where natural occurrence of stress at specific times of the growing season is predictable, as in the case of low temperatures at the beginning and end of the growing season in the maize-growing geographies of Northern United States and the maize-growing geographies of Canada. The short duration of growth that allows the escape response is also associated with lesser yields due to the naturally short period of growth available. Tolerance, on the other hand, allows the plant to encounter and withstand stress occurring during its growing season, through the use of physiological, morphological, and biochemical mechanisms that protect growth and reproduction under stress. Phenotyping for yield stability under abiotic stress is predominantly targeted toward tolerance to exposure to the stress.

Research on drought tolerance in the plant biotechnology industry employs high-throughput screens in model plant species to identify genes that confer a chosen secondary trait or response associated with stress tolerance, with the goal of employing these genes for the development of commercial seed products with stable yields under stress. A schematic of the process in relation to the associated concept and challenges is presented in Figure 2.1, specifically with regard to the development of

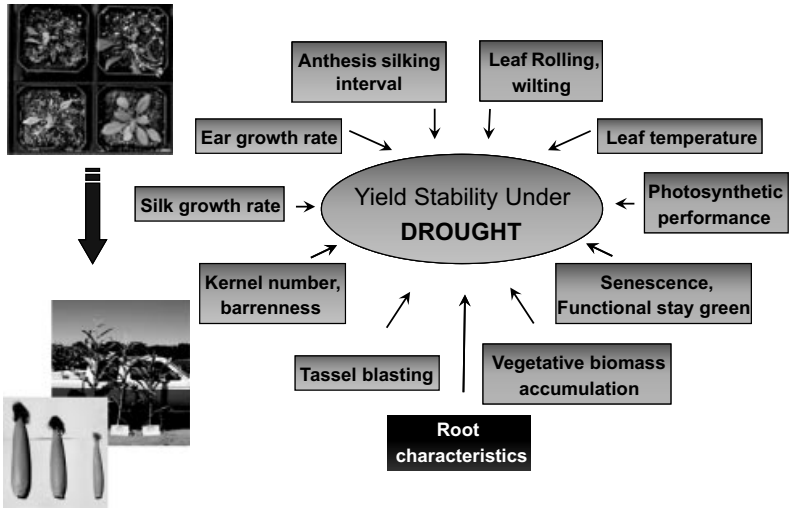

Figure 2.1 High-throughput phenotyping for drought tolerance – from model systems to maize. High-throughput phenotyping is most commonly applied to a model plant system such as *Arabidopsis*, usually at the vegetative stage in forward and reverse genetics approaches. The genes identified are utilized to modify yield stability under abiotic stress conditions in crop species. The challenge becomes even more daunting when one recognizes the fact that yield stability under a stress such as drought is determined by multiple component traits attributable to vegetative, reproductive, and root growth aspects.

drought tolerance in maize. As described in Section 2.2, model plant species such as *Arabidopsis* are used in high-throughput screens under controlled environments, wherein drought stress is usually applied at the vegetative stage, to identify genes that confer tolerance to stress. Plant treatments in these screens normally involve either (1) the withholding of water over a period of time to determine if the gene involved allows the plant to stay turgid for a longer period or to wilt slower, than others, or (2) the withholding of water to a soil moisture content that is close to permanent wilting point, and then rewatering to identify the effect of the gene on plant survival after the drastic stress. Genes identified from such screens are then transferred to commercial crop plants and optimized there, with the aim of providing tolerance to the crop plant under field drought such that yield stability can be achieved. Yield stability thus achieved is defined at the product level in terms of reduced yield loss upon the occurrence of drought during the growing season in plants harboring the transgene, relative to control plants that do not have the transgene.

There are certain crucial factors to be considered in the process of identifying a drought-relevant gene from a model species screen and applying it to trait improvement in crop species. Depending on the time of occurrence of drought during the growth period, and the varying impact of the stress upon different developmental stages, yield stability under drought stress can be affected to varying degrees by seedling establishment, vegetative tolerance, reproductive tolerance, rooting characteristics, osmotic adjustment, growth regulators, rate of senescence, and remobilization of reserves [56]. The need for high throughput in the screening of large numbers of transgenic plants to identify genes of relevance to drought tolerance necessitates the adherence to small plant sizes and short growth durations. Thus, the high-throughput nature of the screen itself imposes constraints both upon the growth stage of the plant at which drought stress can be imposed and upon the species of plants that can be phenotyped in a cost- and time-efficient manner. Furthermore, a screen aimed at gene discovery is confined, due to its high-throughput nature, to measuring only a proportion of the morphophysiological component traits known to be influenced by drought, and heritability of the trait might not always be a consideration in the choice of the traits to be measured. Finally, screens are conducted in pots under controlled environments, where pot-bound roots experience drought stress at a rate of occurrence that is significantly more acute than the slower natural progression of drought under field conditions. Though direct screening for yield in the field can overcome much of the above limitations of model-system vegetative screens, this will be at the cost of time and space efficiency. Such field screens are also subjected to significantly higher variability of the field environment relative to controlled environment conditions. Although field-based yield screens in commercial crop species are done to a limited extent for gene discovery, model species screens under controlled environments for a vegetative phenotype under drought remain the preferred option for high-throughput gene discovery in most instances. The question that is under considerable discussion at present is the relevance of a screen conducted at the vegetative stage in a model plant species under controlled environments to the prevention of yield loss under drought in crop plant species in the field [2, 57–61].

In addressing this question, first we have to acknowledge the fact that relative to single-gene traits such as herbicide tolerance and insecticide tolerance, abiotic stress tolerance is complex and multigenic, and the final yield under stress is a composite of multiple physiological and biochemical responses integrated over the duration of the crop growing period. The final yield of the crop is the composite result of the integrated contributions of these various responses or secondary traits, expressed over time, under varying drought environments. Although a single response pattern cannot be expected to be highly correlated with yield under all drought environments, the physiologically relevant integrators of the effects of drought are the water content and the water potential of plant tissues [59]. Both of these are measurable, and they integrate the effects of drought into the ability to retain turgor at the tissue or the whole-plant level. The utilization of a visible wilting or turgid phenotype at the vegetative stage as a screening criterion in high-throughput controlled environment screens, therefore, is indicative of such integrated response of the plant to drought stress. Under full-cycle growth in the field, this same capacity to maintain tissue- and whole-plant turgor or water potential under drought stress impacts critical responses and secondary traits known to be associated with yield under stress and having high heritability, for example, leaf expansion rate and anthesis-silking interval, as applied to maize [62, 63].

The response to low water potential has been elaborated at the organism and cellular levels by [58] building upon the original concept of stress avoidance and stress tolerance described by Levitt in 1972 [64]. According to this elaboration, stress avoidance happens at low stress levels where plant tissue avoids low water potential and decreased water content by attempting to maintain a balance between water uptake and water loss through stomatal closure and increased root/shoot ratio. If the stress becomes more and the plant tissue cannot avoid low water potential, dehydration avoidance or dehydration tolerance mechanisms are used. In dehydration avoidance, stress responses such as osmotic adjustment and cell wall hardening are used to maintain a high water content despite a reduced water potential. In dehydration tolerance, the tissue employs mechanisms to tolerate cellular damage caused by the water loss, including protective solutes and proteins, metabolic changes, and detoxification of reactive oxygen species. It is important to note that molecular events initiated by water stress do not exclusively fall into one or the other of the above categories of avoidance and tolerance, and that avoidance and tolerance do not occur in a linear progression upon the onset of stress.

Thus, the water content and water potential of plant tissues under drought are influenced by morphophysiological factors such as rooting characteristics, stomatal conductance, and hormonal effects, along with biochemical processes such as osmotic adjustment, membrane and macromolecular protection, antioxidative defense, and signaling. Individual molecular events are involved in determining the expression of these tissue-level morphophysiological and biochemical events, which are integrated into the expression of the phenotype identifiable as wilted or turgid at the whole-plant level. Thus, a gene or a molecular event that is capable of affecting the tissue- and cellular-level phenomena identified above will produce measurable wilting or turgidity, and a screen for such a phenotype identifies genes

involved in the expression of those responses that integrate the effects of water content and water potential of the tissue.

Genes discovered to be associated with a slow-wilting phenotype might not necessarily translate immediately to yield under stress. The impact of a gene on tolerance to drought and on yield stability is determined by the secondary trait affected by it, the heritability of this secondary trait and its contribution to final yield, the intensity of the gene's effect on the secondary trait, and the timing and tissue-type of expression of the gene. Thus, identification of a gene's association with a slow-wilting phenotype may not in itself ensure a commercial product with yield stability under drought. Information on the secondary trait impacted by the gene, the extent of contribution of the specific secondary trait to final yield, and the requisite timing and tissue-type of expression of the gene for optimal manifestation of the secondary trait in question are all critical to the design of the plant transformation construct that maximizes the potential of the gene to impact yield under stress. In instances where the impact of the gene is on secondary traits with small but heritable contributions to final yield, combining two or more of such genes can capture any additive effects. In instances where definitive spatial and temporal modulation of gene expression is necessary, promoter optimization becomes important.

Though a visual or quantitative wilting screen is the most common high-throughput technique applied to model species for discovery of genes associated with drought tolerance, several of the secondary traits that respond to specific molecular events upon the onset of drought, and that integrate the effects of water content and water potential, can be measured quantitatively or qualitatively. Measurement of these secondary traits associated with drought tolerance can be incorporated into screens or into functional characterization efforts to provide relevant information to help determine the functional effect of the gene. Studies that have evaluated the relevance of specific physiological secondary traits for the response to drought conditions have been reviewed recently by Cattivelli *et al*. [4]. The traits evaluated include stomatal conductance, leaf temperature, photosynthetic capacity, timing of phenological phases, anthesis-silking interval in maize, starch availability during ovary/embryo development, partitioning and stem reserve utilization, stay green, single plant leaf area, rooting depth, cuticular resistance and surface roughness, osmotic adjustment, membrane composition, antioxidative defense, and accumulation of stress-related proteins.

Relating the effect of a gene or a genotype to its associated phenotype or secondary trait with sufficient confidence and precision requires carefully planned experimentation and can be done in controlled environments. Determining the contribution of a given secondary trait to final yield is more challenging and requires carefully planned field experiments under managed drought stress and under naturally occurring drought in targeted environments. Such field experimentation attempts to integrate responses to drought over the entire growing season and allows the characterization of gene or genotype effect, and genotype $\times$ environment (G $\times$ E) interaction. Ideally, these experiments should include the measurement of as many secondary traits associated with yield stability as known.

Both vegetative and reproductive screens under controlled environments are employed in gene discovery in much of the plant biotechnology industry addressing

abiotic stress tolerance, including companies such as DuPont Agricultural Biotechnology, Wilmington, DE, USA, and its subsidiary, Pioneer Hi-Bred Inc., Johnston, IA, USA; Monsanto, St Louis, MO, USA; BASF Plant Science, Research Triangle Park, NC, USA, and its subsidiary, Crop Design, Ghent, Belgium; and Ceres, Thousand Oaks, CA, USA. In the public sector, the Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany; the Plant Accelerator, Adelaide, Australia; and the Australian Plant Phenomics Facility, Canberra, Australia are key centers engaged in high-throughput plant phenotyping. Phenomics technologies used in gene discovery and in functional gene characterization, under both controlled and field environments, continue to evolve and have been recently summarized [65, 66]. The bioimaging company LemnaTec GmbH provides imaging and quantification capabilities for high-throughput plant phenotyping in controlled environments.

2.4
Recent Breakthroughs in Abiotic Stress Tolerance in the Plant Biotechnology Industry

Over the past two decades, there have been several studies on the use of single genes for the transgenic modification of abiotic stress tolerance, and a majority of these have been on model plant species, such as *Arabidopsis*. The genes used to modify abiotic stress tolerance address several different stress-related secondary responses and biochemical pathways including osmolyte synthesis and osmotic adjustment, hormonal regulation, antioxidants, transcription factors and other signaling genes, dehydrins, and so on [67, 68]. Almost all of the published studies report transgene evaluation under controlled environments with a focus on the vegetative phenotype. Only in very few instances has the phenotype conferred by the transgene been evaluated in the field in commercial crop species over full-growth cycle. These include studies both from the public sector and from the private sector [31, 69–71].

In the public sector, Xiao *et al*. identified Late Embryogenesis Abundant gene from rice, *OS LEA3-1*, on the basis of its drought-induced expression in microarray studies, and overexpressed this in rice from both a drought-inducible and a constitutive promoter, to obtain a drought-tolerant phenotype with higher grain yield than the wild type under drought stress in the field [69]. In another instance, Oh *et al*. studied genes containing the *APETALA2 (AP2)* domain in rice and identified several with stress-inducible expression [71]. They focused on two of the identified genes for functional characterization, and overexpression of *AP37* in rice from a constitutive promoter conferred a drought-tolerant phenotype with enhanced yield compared to the wild-type control in the field under severe drought stress. The yield of the transgenic plants was on par with that of the wild-type control under normal growth conditions of optimal soil moisture.

A majority of the research on this topic in the private sector remains unpublished as yet. Transgenic evaluation of the plant *NUCLEAR FACTOR (NF-Y)B* and that of the bacterial RNA chaperone, the *COLD SHOCK PROTEIN B* or *CSPB*, from Monsanto are two published studies on drought tolerance, where single transgenes

transformed into commercial crop species have been evaluated for yield and associated traits under drought stress in the field [31, 70]. An application for *NF-YB* to drought was identified from screens for drought tolerance using over 1500 *Arabidopsis* transcription factors in the Mendel Biotechnology-Monsanto collaboration [31]. Overexpression of the *Arabidopsis NF-YB1* gene in *Arabidopsis* from the constitutive cauliflower mosaic virus *35S* promoter was found to confer drought tolerance in the form of reduced wilting, increased survival, enhanced water potential, and photosynthesis under drought-stressed conditions, compared to the wild-type nontransgenic control. The maize homologue of the *Arabidopsis* gene, *ZM NF-YB2*, when constitutively expressed in maize from the rice actin promoter conferred enhanced drought tolerance as observed by less wilting and increased seedling survival in the greenhouse. In the field, transgenic maize plants showed less leaf rolling, higher chlorophyll index, higher photosynthesis rate, cooler leaf temperature, higher stomatal conductance, and enhanced yield compared to nontransgenic check plants.

Castiglioni *et al*. at Monsanto reported the effect of bacterial *COLD SHOCK PROTEINS*, *CSPA* and *CSPB*, on stress tolerance under controlled environments in *Arabidopsis* and rice, and both under controlled environments and in the field in maize [70]. In *Arabiodpsis*, both proteins showed enhanced growth when subjected to cold stress, and in rice, both showed enhanced plant height, an indication of better growth, under cold and heat treatment. *CSPB* also produced similar positive effects on rice under drought treatment. When *CSPB* was transformed into maize, seedling vegetative performance was improved compared to nontransformed control plants when subjected to drought stress, and this was demonstrated by improved leaf elongation rate, a measure of growth, chlorophyll content, and photosynthetic performance. One *CSPB*-expressing maize event was tested in the field across three different hybrid backgrounds, two different stress regimes (stress during vegetative phase and stress during reproductive phase), and five replicated locations. The tested event consistently outyielded the nontransgenic controls in a majority of these treatments. In 3-year trials, it showed yield advantage over the control in a variety of stress environments where yield reductions ranged from 20 to 80% due to the occurrence of some level of water stress.

The above results indicate that yield stability under drought can be achieved either through single-gene modifications, where the transgene might constitute enhancement of inherent variability in the crop species, or through the introduction of new variability hitherto nonexistent in the crop species. The yield advantages reported in these published studies are high, although consistent adherence to these early published results across time, multiple locations, and different genetic backgrounds remains yet to be proved. Even if the yield advantages do not remain as high as the reported early results, the fact remains that drought tolerance can be transgenically modified by single genes, and careful studies should allow the combination of smaller effects of more than one such "positive" gene in order to capture additive effects. One aspect that continues to stand out and that needs constant consideration in transgenic research on drought tolerance is that genes capable of conferring drought tolerance under water-limited environments should ideally show yields

comparable to control plants under well-watered environments. Failure to satisfy this condition will prevent the wider deployment of drought-tolerant crop products and necessitate confinement to regions of extreme drought occurrence.

2.5 Conclusions and Future Perspectives

A vast volume of scientific literature over the past three decades has addressed gene discovery for abiotic stress research, focusing on model plant species, primarily *Arabidopsis*. More recently, the complexity and multigenic nature of abiotic stress tolerance has directed this research into high-throughput gene discovery and gene evaluation. The very high-throughput nature of the present research necessitates the adherence to short-duration small-sized model plant species to enable economies of space and time for gene discovery, while recognizing the challenges of translating functional gene efficacy from model species to field relevance and yield in commercial crop plants. Single-gene functional efficacy has been reported to confer changes in morphophysiological, biochemical, and molecular traits associated with tolerance to abiotic stresses, essentially with relevance to the vegetative performance of the plant. Most recent research has also seen the translation of this single-gene functional efficacy to yield improvement in the field under drought. Challenges remain in achieving yield stability that can be transferable across more than one growing environment, and tolerance that can address stress occurring at more than one physiological growth phase during the cropping season.

References

1 Campos, H., Cooper, M., Habben, J.E., Edmeades, G.O., and Schussler, J.R. (2004) *Field Crops Res.*, **90**, 19–34.
2 Edmeades, G.O., McMaster, G.S., White, J.W., and Campos, H. (2004) *Field Crops Res.*, **90**, 5–18.
3 Hammer, G., Cooper, M., Tardieu, F., Welch, S., Walsh, B., van Eeuwijk, F., Chapman, S., and Podlich, D. (2006) *Trends Plant Sci.*, **11**, 587–593.
4 Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A.M., Francia, E., Marè, C., Tondelli, A., and Stanca, A.M. (2008) *Field Crops Res.*, **105**, 1–14.
5 Cooper, M., van Eeuwijk, F.A., Hammer, G.L., Podlich, D.W., and Messina, C. (2009) *Curr. Opin. Plant Biol.*, **12**, 231–240.
6 Mittler, R. and Blumwald, E. (2010) *Annu. Rev. Plant. Biol.*, **61**, 443–462. Review.
7 Forster, B.P., Ellis, R.P., Moir, J., Talame, V., Sanguineti, M.C., Tuberosa, R., This, D., Teulat-Merah, B., Ahmed, I., Mariy, SAE., Bahri, H., El Ouahabi, M., Zoumarou-Wallis, N., El-Fellah, M., and Ben Salem, M. (2004) *Ann. Appl. Biol.*, **144**, 157–168.
8 Collins, N.C., Tardieu, F., and Tuberosa, R. (2008) *Plant Physiol.*, **147**, 469–486.
9 Alexandrov, N.N., Brover, V.V., Freidin, S., Troukhan, M.E., Tatarinova, T.V., Zhang, H., Swaller, T.J., Lu, Y.P., Bouck, J., and Flavell, R.B., and Feldmann, K.A. (2009) *Plant Mol. Biol.*, **69**, 179–194.
10 Zhu, J.K. (2001) *Curr. Opin. Plant Biol.*, **4**, 401–406. Review.
11 Peters, J.L., Cnudde, F., and Gerats, T. (2003) *Trends Plant Sci.*, **8**, 484–491. Review.

12 Alonso, J.M. and Ecker, J.R. (2006) *Nat. Rev. Genet.*, **7**, 524–536. Review.

13 Kondou, Y., Higuchi, M., and Matsui, M. (2010) *Annu. Rev. Plant Biol.*, **61**, 373–393. Review.

14 Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J.K. (1997) *Plant Cell*, **9**, 1935–1949.

15 Huang, S., Cerny, R.E., Bhat, D.S., and Brown, S.M. (2001) *Plant Physiol.*, **125**, 573–584.

16 Griffith, M., Timonin, M., Wong, A.C., Gray, G.R., Akhter, S.R., Saldanha, M., Rogers, M.A., Weretilnyk, E.A., and Moffatt, B. (2007) *Plant Cell Environ.*, **30**, 529–538.

17 Kant, S., Bi, Y.M., Weretilnyk, E., Barak, S., and Rothstein, S.J. (2008) *Plant Physiol.*, **147**, 1168–1180.

18 Amtmann, A. (2009) *Mol. Plant.*, **2**, 3–12.

19 Taji, T., Sakurai, T., Mochida, K., Ishiwata, A., Kurotani, A., Totoki, Y., Toyoda, A., Sakaki, Y., Seki, M., Ono, H., Sakata, Y., Tanaka, S., and Shinozaki, K. (2008) *BMC Plant Biol.*, **8**, 115.

20 Du, J., Huang, Y.P., Xi, J., Cao, M.J., Ni, W.S., Chen, X., Zhu, J.K., Oliver, D.J., and Xiang, C.B. (2008) *Plant J.*, **56**, 653–664.

21 Frank, W., Ratnadewi, D., and Reski, R. (2005) *Planta*, **220**, 384–394.

22 Richardt, S., Timmerhaus, G., Lang, D., Qudeimat, E., Corrêa, L.G., Reski, R., Rensing, S.A., and Frank, W. (2010) *Plant Mol. Biol.*, **72**, 27–45.

23 Schween, G., Egener, T., Fritzowsky, D., Granado, J., Guitton, M.C., Hartmann, N., Hohe, A., Holtorf, H., Lang, D., Lucht, J.M., Reinhard, C., Rensing, S.A., Schlink, K., Schulte, J., and Reski, R. (2005) *Plant Biol. (Stuttg)*, **7**, 228–237.

24 Lang, D., Zimmer, A.D., Rensing, S.A., and Reski, R. (2008) *Trends Plant Sci.*, **13**, 542–549. Review.

25 Opanowicz, M., Vain, P., Draper, J., Parker, D., and Doonan, J.H. (2008) *Trends Plant Sci.*, **13**, 172–177.

26 Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., and Yu, G. (2000) *Science*, **290**, 2105–2110.

27 Riechmann, J.L. and Ratcliffe, O.J. (2000) *Curr. Opin. Plant Biol.*, **3**, 423–434. Review.

28 Zhang, J.Z. (2003) *Curr. Opin. Plant Biol.*, **6**, 430–440. Review.

29 Gutterson, N. and Zhang, J.Z. (2004) *Curr. Opin. Plant Biol.*, **7**, 226–230. Review.

30 Zhang, J.Z., Creelman, R.A., and Zhu, J.K. (2004) *Plant Physiol.*, **135**, 615–621.

31 Nelson, D.E., Repetti, P.P., Adams, T.R., Creelman, R.A., Wu, J., Warner, D.C., Anstrom, D.C., Bensen, R.J., Castiglioni, P.P., Donnarummo, M.G., Hinchey, B.S., Kumimoto, R.W., Maszle, D.R., Canales, R.D., Krolikowski, K.A., Dotson, S.B., Gutterson, N., Ratcliffe, O.J., and Heard, J.E. (2007) *Proc. Natl. Acad. Sci., USA*, **104**, 16450–16455.

32 Century, K., Reuber, T.L., and Ratcliffe, O.J. (2008) *Plant Physiol.*, **147**, 20–29. Review.

33 Ormenese, S., de Almeida Engler, J., De Groodt, R., De Veylder, L., Inzé, D., and Jacqmard, A. (2004) *Ann. Bot.*, **93**, 575–580.

34 Inzé, D. and De Veylder, L. (2006) *Annu. Rev. Genet.*, **40**, 77–105.

35 De Block, M., Verduyn, C., De Brouwer, D., and Cornelissen, M. (2005) *Plant J.*, **41**, 95–106.

36 Vanderauwera, S., De Block, M., Van de Steene, N., van de Cotte, B., Metzlaff, M., and Van Breusegem, F. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 15150–15155.

37 Zheng, Z., Xu, X., Crosley, R.A., Greenwalt, S.A., Sun, Y., Blakeslee, B., Wang, L., Ni, W., Sopko, M.S., Yao, C., Yau, K., Burton, S., Zhuang, M., McCaskill, D.G., Gachotte, D., Thompson, M., and Greene, T.W. (2010) *Plant Physiol.*, **153**, 99–113.

38 Wang., Y., Ying, J., Kuzma, M., Chalifoux, M., Sample, A., McArthur, C., Uchacz, T., Sarvas, C., Wan, J., Dennis, D.T., McCourt, P., and Huang, Y. (2005) *Plant J.*, **43**, 413–424.

39 Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S., and McCourt, P. (1996) *Science.*, **273**, 1239–1241.

40 Lassner, M. and Bedbrook, J. (2001) *Curr. Opin. Plant Biol.*, **4**, 152–156.

41 Kurek, I., Chang, T.K., Bertain, S.M., Madrigal, A., Liu, L., Lassner, M.W., and Zhu, G. (2007) *Plant Cell*, **19**, 3230–3241.

42 Kollipara, K.P., Saab, I.N., Wych, R.D., Lauer, M.J., and Singletary, G.W. (2002) *Plant Physiol.*, **129**, 974–992.

43 Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., and Harper, J.F. (2002) *Plant Physiol.*, **130**, 2129–2141.

44 Zinselmeier, C., Sun, Y., Helentjaris, T., Beatty, M., Yang, S., Smith, H., and Habben, J. (2002) *Field Crops Res.*, **75**, 111–121.

45 Troukhan, M., Tatarinova, T., Bouck, J., Flavell, R.B., and Alexandrov, N.N. (2009) *OMICS*, **13**, 139–151.

46 Timperio, A.M., Egidi, M.G., and Zolla, L. (2008) *J. Proteomics*, **71**, 391–411.

47 Wienkoop, S., Baginsky, S., and Weckwerth, W. (2010) *J. Proteomics*, **73**, 2239–2248.

48 Mazur, B.J. (2003) *Nat. Biotechnol.*, **21**, 875–876.

49 Saito, K. and Matsuda, F. (2010) *Annu. Rev. Plant Biol.*, **61**, 463–489.

50 Harrigan, G.G., Stork, L.G., Riordan, S.G., Ridley, W.P., Macisaac, S., Halls, S.C., Orth, R., Rau, D., Smith, R.G., Wen, L., Brown, W.E., Riley, R., Sun, D., Modiano, S., Pester, T., Lund, A., and Nelson, D. (2007) *J. Agric. Food Chem.*, **55**, 6169–6176.

51 Wang, D., Guo, Y., Wu, C., Yang, G., Li, Y., and Zheng, C. (2008) *BMC Genomics*, **27**, **9**, 44.

52 Xue, T., Wang, D., Zhang, S., Ehlting, J., Ni, F., Jakab, S., Zheng, C., and Zhong, Y. (2008) *BMC Genomics*, **9**, 550.

53 Hoth, S., Morgante, M., Sanchez, J.P., Hanafey, M.K., Tingey, S.V., and Chua, N.H. (2002) *J. Cell Sci.*, **115**, 4891–4900.

54 Hazen, S.P., Pathan, MS., Sanchez, A., Baxter, I., Dunn, M., Estes, B., Chang, H.S., Zhu, T., Kreps, J.A., and Nguyen, H.T. (2005) *Funct. Integr. Genomics*, **5**, 104–116.

55 Sade, N., Vinocur, B.J., Diber, A., Shatil, A., Ronen, G., Nissan, H., Wallach, R., Karchi, H., and Moshelion, M. (2009) *New Phytol.*, **181**, 651–661.

56 Barker, T., Campos, H., Cooper, M., Dolan, D., Edmeades, G., Habben, J., Schussler, J., Wright, D., and Zinselmeier, C. (2005) *Plant Breed. Rev.*, **25**, 173–253.

57 Sinclair, T.R. and Purcell, L.C. (2005) *J. Exp. Bot.*, **56**, 2777–2782.

58 Verslues, P.E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., and Zhu, J.K. (2006) *Plant J.*, **45**, 523–539.

59 Jones, H.G. (2007) *J. Exp. Bot.*, **58**, 119–130.

60 Salekdeh, G.H., Reynolds, M., Bennett, J., and Boyer, J. (2009) *Trends Plant Sci.*, **14**, 488–496.

61 Munns, R., James, R.A., Sirault, X.R., Furbank, R.T., and Jones, H.G. (2010) *J. Exp. Bot.*, **61**, 3499–3507.

62 Salah, H. and Tardieu, F. (1997) *Plant Physiol.*, **114**, 893–900.

63 Welcker, C., Boussuge, B., Bencivenni, C., Ribaut, J.M., and Tardieu, F. (2007) *J. Exp. Bot.*, **58**, 339–349.

64 Levitt, J. (1972) *Responses of Plants to Environmental Stresses*, Academic Press, New York, NY.

65 Furbank, R.T. (2009) *Funct. Plant Biol.*, **36**, v–v10.

66 Berger, B., Parent, B., and Tester, M. (2010) *J. Exp. Bot.*, **61**, 3519–3528.

67 Bhatnagar-Mathur, P., Vadez, V., and Sharma, K.K. (2008) *Plant Cell Rep.*, **27**, 411–424.

68 Ashraf, M. (2010) *Biotech. Adv.*, **28**, 169–183.

69 Xiao, B., Huang, Y., Tang, N., and Xiong, L. (2007) *Theor. Appl. Genet.*, **115**, 35–46.

70 Castiglioni, P., Warner, D., Bensen, R.J., Anstrom, D.C., Harrison, J., Stoecker, M., Abad, M., Kumar, G., Salvador, S., D'Ordine, R., Navarro, S., Back, S., Fernandes, M., Targolli, J., Dasgupta, S., Bonin, C., Luethy, M.H., and Heard, J.E. (2008) *Plant Physiol.*, **147**, 446–455.

71 Oh, S.J., Kim, Y.S., Kwon, C.W., Park, H.K., Jeong, J.S., and Kim, J.K. (2009) *Plant Physiol.*, **150**, 1368–1379.

3
Generation and Scavenging of Reactive Oxygen Species in Plants under Stress

Sarvajeet Singh Gill, Lamabam Peter Singh, Ritu Gill, and Narendra Tuteja

Reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$), and perhydroxyl radicals ($HO_2^{\bullet -}$) are constantly produced as by-products of various metabolic processes in plants and continuously reduced/scavenged by plant antioxidative defense system to maintain at a certain steady-state levels. Any disruption in this delicate balance between ROS generation and reduction/scavenging leads to high accumulation in plant cells, which causes oxidative stress. Plants counteract ROS toxicity through enzymatic antioxidant systems comprising a range of ROS scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), guaiacol peroxidase (GPOX), glutathione reductase (GR), and glutathione *S*-transferase (GST), and nonenzymatic low molecular metabolites, such as ascorbic acid (ASH), glutathione (GSH), α-tocopherol, proline, carotenoids, and flavonoids. Various biotic and abiotic stresses such as plant diseases, drought, salinity, extreme temperatures, excess light, pollutants, nutrient deficiency, and so on disturb the equilibrium of ROS production and scavenging, eventually leading to overproduction and higher accumulation of ROS. High amount of ROS in plant cells affects various cellular functions through damaging nucleic acids, protein oxidation, and lipid peroxidation, eventually resulting in cell death. ROS toxicity resulted from various biotic and abiotic stress factors is considered to be one of the major causes of low crop productivity worldwide. Despite this, it has also become clear that ROS play an important signaling role in plants, controlling various processes such as cellular growth, control of stomata closing, plant–harmful/beneficial microbe interactions, programmed cell death, and stress responses, and can also initiate responses such as new gene expression.

3.1
Introduction

Stress, in the context of plants, is "any unfavorable condition or substance that affects or blocks a plant's metabolism, growth, or development." Factors that induce

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stress to plants can be of two types: "biotic," resulting from living organisms, such as fungi, bacteria, viruses, pests, herbivores, and so on, and "abiotic," resulting from nonliving factors, such as drought, salinity, extreme temperatures, excess light, pollutants, xenobiotics, ultraviolet radiation, ozone, hypoxia, nutrient deficiency, and so on. The balance between tolerance and sensitivity of a particular plant may determine whether a stress factor has a positive or negative effect on that plant [1]. As the stress factors are variable, the mechanisms of damage and, consequently, the plant signaling and metabolic responses differ from each other. Yet, plants respond to all these stresses by increasing generation/production of reactive oxygen species (ROS), although their identity and compartment of origin may certainly differ [2, 3].

Reactive oxygen species comprise $O_2^{\bullet -}$, H_2O_2, $^1O_2$, $OH^{\bullet}$, $HO_2^{\bullet -}$, ROOH, $ROO^{\bullet}$, and $RO^{\bullet}$. ROS are the spin-off, invariably generated by plants during various metabolic processes in different cellular compartments, such as chloroplast, mitochondria, peroxisomes, cytosol, plasma membrane, and apoplastic space [4–6]. Under stable/normal conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms [7]. The equilibrium between the production and scavenging of ROS may be disturbed by various biotic and abiotic stress factors, such as salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, pollutants, herbicides, and pathogen attacks. These disturbances in equilibrium lead to sudden increase in intracellular ROS level, which can cause significant damage to cell structures. It has been estimated that 1–2% of O_2 consumption leads to the formation of ROS in plant tissues [8]. Through a variety of reactions, $O_2^{\bullet -}$ leads to the formation of H_2O_2, $OH^{\bullet}$, and other ROS. ROS are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates, and DNA, which eventually results in cell death. Accumulation of ROS as a result of various environmental stresses is a major cause of loss of crop productivity worldwide [9–15]. High ROS production and accumulation affects many cellular functions by damaging nucleic acids, oxidizing proteins, and lipid peroxidation (LPO) [7].

Conversely, now it has become apparent that ROS also play an important signaling role in plants, controlling various processes such as cellular growth, stomata closing [16], plant–pathogen interactions [10], programmed cell death [17], and stress responses [18, 19]. ROS can also initiate responses such as new gene expression, regulate and involve in legume–*rhizobia* symbiosis [20, 21], and establish both endo- and ectomycorrhiza [22]. It is the evolution of highly efficient scavenging mechanisms that most likely enabled plant cells to overcome ROS toxicity and led to the use of several of these ephemeral reactive molecules as signal transducers. The recent identification of ROS-generating enzymes, such as the plant homologue of respiratory burst NADPH oxidases, has led to the demonstration that plant cells, similar to mammalian cells, can initiate and most likely amplify ROS production for the purpose of signaling. Localized ROS production in organelles such as plastids, mitochondria, and peroxisomes may also initiate signaling cascades [23]. It is important to note that whether ROS will act as damaging, protective, or signaling factors depends on the delicate equilibrium between ROS production and scavenging

at the proper site and time [24]. Stress-induced ROS accumulation is counteracted by enzymatic antioxidant systems that include a variety of scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), GPX, glutathione *S*-transferase (GST), and catalase (CAT), and nonenzymatic low molecular metabolites, such as ascorbic acid (ASH), glutathione (GSH), α-tocopherol, carotenoids, and flavonoids [15, 25]. Besides, proline has also been added to an elite list of nonenzymatic antioxidants that microbes, animals, and plants need to counteract the inhibitory effects of ROS [26]. The above-mentioned antioxidants are found in almost all cellular compartments, signifying the importance of ROS detoxification for cellular survival [15]. Plant stress tolerance may therefore be improved by the enhancement of *in vivo* levels of antioxidant enzymes [4]. At present, it has also been shown that ROS influence the expression of a number of genes and signal transduction pathways, suggesting that cells have evolved strategies to use ROS as biological stimuli and signals that activate and control various genetic stress–response programs [27]. Recently, it has become evident that plants actively produce ROS, which may control many different physiological processes such as biotic and abiotic stress responses, pathogen defense, and systemic signaling [4].

3.2 ROS Production

ROS are perpetually produced through cellular metabolism and plant cells are well equipped with antioxidants and scavenging enzymes to keep their levels in check under normal growth conditions. Biotic and abiotic stresses can increase the rate of ROS production and collectively with the compartment-specific (down) regulation of the cells' antioxidant capacity, it can lead to significant ROS accumulation in plant cells. Till date, numerous studies have well documented the harmful effects of ROS on cellular components [5], and now, a role in plant signaling has also been firmly established [28].

Chloroplasts are the prime source of ROS in photosynthetic tissues due to their capacity to produce high amounts of $O_2^{\bullet -}$ and H_2O_2, especially during reduced rate of photosynthetic carbon fixation, which is a typical condition during abiotic stresses [29]. Oxygen generated in the chloroplasts during photosynthesis can accept electrons passing through the photosystems, thus resulting in the formation of $O_2^{\bullet -}$. Therefore, the presence of ROS producing centers, such as triplet chlorophyll and electron transport chain (ETC) in PSI and PSII, makes chloroplasts a major site of ROS ($O_2^{\bullet -}$, $^1O_2$, and H_2O_2 production. Various abiotic stresses such as excess light, drought, salt stress, and CO_2-limiting conditions enhance the production of ROS in chloroplasts. Normally, the electron flow from the excited photosystem centers is directed to $NADP^+$, which is reduced to NADPH. Then, it enters the Calvin cycle and reduces the final electron acceptor, CO_2. In case of overloading conditions of the ETC, a part of the electron flow is diverted from ferredoxin to O_2, reducing it to $O_2^{\bullet -}$ via Mehler reaction [30, 31]. Later studies reveal

that the acceptor side of ETC in PSII also provides sides (QA, QB) with electron leakage to O_2 producing $O_2^{\bullet -}$ [32]. $^1O_2$ is a natural by-product of photosynthesis, mainly formed at PSII even under low light conditions [33]. On the external, "stromal" membrane surface, $O_2^{\bullet -}$ is spontaneously dismutated to H_2O_2 by Cu/Zn-SOD [32]. Recent researches have linked chloroplast-produced ROS with the hypersensitive response [34]. Chloroplast-produced ROS have been shown to be capable of transmitting the spread of wound-induced PCD through maize tissue [35]. The expression of animal antiapoptotic Bcl-2 family members in transgenic tobacco has revealed the involvement of chloroplast in oxidative stress-induced PCD [36]. It has been shown that in *Arabidopsis thaliana* cell suspension cultures, the cells contain well-developed, functional chloroplasts when grown in the light, but not in the dark, and can be used as model systems to study PCD. In a study, treatment with antioxidant of light-grown cultures resulted in increased apoptotic-like PCD induction, which suggests the involvement of chloroplast-produced ROS apoptotic-like PCD regulation. It has been suggested that chloroplasts can play a significant role in apoptotic-like PCD regulation [37].

Mitochondria are the major source of ROS in nonphotosynthetic tissues, but in a green cell of a plant, their contribution is considered small in comparison to chloroplasts [3]. The redox status of the mitochondrial ETC is an important indicator of the cell energy status, and ROS, especially $O_2^{\bullet -}$ from complexes I and III, and the reduction status of the ubiquinone pool are integral parts of this monitoring system [38]. The mitochondrial ETC harbors electrons with sufficient free energy that directly reduce O_2, which is considered an unavoidable primary source of mitochondrial ROS generation and a necessary accompaniment to aerobic respiration [39]. However, ROS production in mitochondria takes place under normal respiratory conditions that can be enhanced in response to various biotic and abiotic stress conditions [4]. $O_2^{\bullet -}$ in aqueous solution is moderately reactive, but it can be further reduced by SOD dismutation to H_2O_2 [40–42]. It has been estimated that about 1–5% of mitochondrial O_2 consumption leads to H_2O_2 production [41]. This H_2O_2 can react with reduced Fe^{2+} and Cu^{+} to produce highly toxic $OH^{\bullet}$, and these uncharged $OH^{\bullet}$ can penetrate membranes and leave the mitochondrion [39, 42, 43]. Peroxidation of mitochondrial membrane PUFA is initiated by the abstraction of a hydrogen atom by ROS, especially by $OH^{\bullet}$. This leads to the formation of cytotoxic lipid aldehydes, alkenals, and hydroxyalkenals, such as the much studied 4-hydroxy-2-nonenal and malondialdehyde (MDA). Once LPO products are formed, they can cause cellular damage by reacting with proteins, other lipids, and nucleic acids. Key oxylipins and smaller, lipid-derived reactive electrophile species may also be produced from LPO [44]. It has also been noted that UV-C exposure induces quick appearance of ROS in the protoplasts, which is restricted to chloroplasts and mitochondria. It is suggested that the mitochondrial transmembrane potential loss and the changes in distribution and mobility of mitochondria, as well as the production of ROS, play important roles during UV-induced plant PCD [45]. It is a well-established fact that abiotic stresses strongly affect the plant cell bioenergetics. Plant mitochondria may control ROS generation by means of energy dissipating systems. Thereby, mitochondria may play a

significant central role in cell adaptation to abiotic stresses, which are known to induce oxidative stress at cellular level [4].

Peroxisomes are also probably one of the major sites of intracellular ROS production. They contain several oxidases that produce H_2O_2 and $O_2^{\bullet -}$ as by-products of the reactions they catalyze. The photorespiratory glycolate oxidase is located in peroxisomes, its function is especially relevant during abiotic stresses, which are often accompanied by stomatal closure, and the resulting decrease in gas exchange leads to reduction in carbon dioxide availability for Rubisco, followed by increased photorespiration and H_2O_2 production [28]. Peroxisomes, such as mitochondria and chloroplasts, produce $O_2^{\bullet -}$ radicals as a consequence of their normal metabolism. Two sites of $O_2^{\bullet -}$ generation are established in peroxisomes [46]. The first site is in the organelle matrix, where xanthine oxidase (XOD) catalyzes the oxidation of xanthine and hypoxanthine to uric acid [47], and the second site is in the peroxisome membranes dependent on NAD(P)H, where a small ETC is composed of a flavoprotein NADH and cytochrome *b*, and here $O_2^{\bullet -}$ is produced by the peroxisome ETC. Monodehydroascorbate reductase (MDHAR) participates in $O_2^{\bullet -}$ production by peroxisome membranes [46]. The main metabolic processes responsible for the generation of H_2O_2 in different types of peroxisomes are the photorespiratory glycolate oxidase reaction, the fatty acid β-oxidation, the enzymatic reaction of flavin oxidases, and the disproportionation of $O_2^{\bullet -}$ radicals [46, 48]. Intensification of H_2O_2 and $O_2^{\bullet -}$ production in the peroxisomes leads to oxidative damage and possibly cell death [46]. Conversely, it has also been shown that small levels of H_2O_2 and $O_2^{\bullet -}$ act as signal molecules that mediate pathogen-induced PCD in plants [49, 50]. Hence, it has been suggested that peroxisomes should be considered as cellular compartments with the capacity to generate and release important signal molecules such as $O_2^{\bullet -}$, H_2O_2, and $NO^{\bullet}$ into the cytosol, which can contribute to a more integrated communication system among cell compartments [47].

Besides these metabolic ROS sources, hydroxyl radicals ($OH^{\bullet}$) can be formed from H_2O_2 in the presence of redox-active metals through the Fenton reaction or from H_2O_2 and $O_2^{\bullet -}$ through the Haber–Weiss reaction. The extremely reactive $OH^{\bullet}$ radical can run rampant in the cell, causing extensive oxidative damage. Although it is not considered to have signaling function, the products of its reactions can elicit signaling responses, and cells sequester the catalytic metals to metallochaperones efficiently avoiding $OH^{\bullet}$ formation [5, 51]. NADPH oxidases (Rbohs for respiratory burst oxidase homologues) are an important ROS-generating system in plants producing $O_2^{\bullet -}$, which is usually dismutated to hydrogen peroxide (H_2O_2) rapidly [52]. *Arabidopsis* Rboh isoforms have been shown to participate in different processes: RbohC is necessary for root hair tip growth and mechanosensing [53] and RbohD and F function in pathogen defense and abscisic acid (ABA) signal transduction [54]. Recently, the role of Rbohs in heavy metal-induced accumulation of ROS [55] and early response to salt stress [56] has been established. In case of salt stress, the ROS-generating activity was localized to internalized plasma membrane vesicles in contrast to the apoplastic ROS production during biotic interactions, root hair growth, and ABA signaling. In addition, *Arabidopsis* RbohD was demonstrated to be responsible for the

fast-moving ROS signal mediating the systemic acclimation to several abiotic stresses [57]. Other important sources of ROS gaining attention are detoxification reactions catalyzed by cytochrome P450 in cytoplasm and endoplasmic reticulum [58]. ROS are also generated in plasma membrane. A number of other ROS-generating systems, such as pH-dependent cell wall peroxidases, amine oxidases, and oxalate oxidases, are present in apoplast, and being dependent on pH, they are activated by alkaline pH, which in the presence of a reductant produces H_2O_2. Alkalization of apoplast upon elicitor recognition precedes the oxidative burst, and generation of H_2O_2 by a pH-dependent cell wall peroxidase has been proposed as an alternative way of ROS production during biotic stress [59]. It was recently shown that H_2O_2 produced by apoplastic polyamine oxidase can influence the salinity stress signaling in tobacco and can play a role in balancing the plant response between stress tolerance and cell death [60].

3.3 ROS Scavenging

ROS are highly toxic, they can react with cellular components such as lipids, proteins, and nucleic acids, and cause lipid peroxidation, membrane damage, and inactivation of enzymes, thus affecting many physiological processes as well as cell viability. Even so, plants have evolved a complex array of mechanisms to retain low ROS level and avoid the detrimental effects of excessively high ROS concentrations [61]. ROS are rapidly detoxified by various cellular enzymatic and nonenzymatic mechanisms in plants. Oxidative stress occurs when there is a serious imbalance between the production of ROS and the antioxidative defense [62]. The components of antioxidant defense system consist of various soluble (ASH, GSH) and membrane (α-tocopherol) compounds as well as enzymes (SOD, APX, GPX, CAT, MDHAR, DHAR, and glutathione reductase (GR)) [4, 61].

3.3.1 Enzymatic Antioxidants

SODs are the most effective intracellular enzymatic antioxidants, which are ubiquitous in all aerobic organisms and subcellular compartments prone to ROS-mediated oxidative stress. Superoxide dismutases act as the first line of defense against the toxic effects of ROS by dismutating superoxide to H_2O_2 [4, 10]. The upregulation of SODs is implicated in combating oxidative stress caused due to biotic and abiotic stresses and has a critical role in the survival of plants under stressful environments [4]. Significant increase in SOD activity under salt stress has been observed in various plants, namely, mulberry [63], *Cicer arietinum* [64], and *Lycopersicon esculentum* [65]. Pan *et al.* [66] investigated the effects of salt and drought stress on *Glycyrrhiza uralensis* Fisch and observed a significantly increased SOD activity, but an additional Mn-SOD isoenzyme was also detected under only salt stress. Increased SOD activity has also been detected following Cd treatment in

Hordeum vulgare [67], *A. thaliana* [68], *Oryza sativa* [69], *Triticum aestivum* [70], *Brassica juncea* [71], *Vigna mungo* [72], and *C. arietinum* [73]. Similarly, increased SOD activity following drought stress was also observed in three cultivars of *Phaseolus vulgaris* [74], *Alternanthera philoxeroides* [75], and *O. sativa* [76]. Wang and Li [77] studied the effect of water stress on the activities of total leaf SOD and chloroplast SOD in *Trifolium repens* L. and observed a significantly increased SOD activity under water stress. Similar result was reported by Simonovicova *et al.* [78] in *H. vulgare* L. cv. Alfor root tips under Al stress. APX, GPX, and CAT subsequently scavenge H_2O_2; however, unlike CAT, APX requires an ascorbate and GSH regeneration system, the ascorbate–glutathione cycle. Detoxifying H_2O_2 to H_2O by APX occurs by oxidation of ascorbate to MDA, which can be regenerated by MDA reductase (MDAR) using NAD(P)H as reducing equivalents. MDA can spontaneously dismutate into dehydroascorbate. Ascorbate regeneration is mediated by dehydroascorbate reductase (DHAR) driven by the oxidation of GSH to GSSG. Finally, GR can regenerate GSH from GSSG using NAD(P)H as a reducing agent [10]. Enhanced expression of APX in plants has been demonstrated under various stress conditions. Increased leaf APX activity under Cd stress has been reported in *Ceratophyllum demersum* [79], *B. juncea* [71], *T. aestivum* [70], and *V. mungo* [72]. Hsu and Kao [80] reported that pretreatment of *O. sativa* seedlings with H_2O_2 under nonheat shock conditions resulted in an increase in APX activity and protected rice seedlings from subsequent Cd stress. Enhanced activity of APX was also observed in *Anabaena doliolum* under salt stress [81], in three cultivars of *P. vulgaris* [74] and *Picea asperata* [82] under water stress, and in *H. vulgare* L. cv. Alfor root tips exposed to Al stress [78]. Sharma and Dubey [76] found that mild drought-stressed plants had higher chloroplastic APX activity than control-grown plants, but the activity declined at the higher level of drought stress. Akin to APX, GPX also detoxifies H_2O_2 to H_2O, but uses GSH directly as a reducing agent. The GPX cycle is closed by regeneration of GSH from GSSG by GR [10]. Abiotic stress increases GPX activity in cultivars of *Capsicum annuum* plants [83], but decreases in roots and causes no significant change in the leaves of Cd-exposed *Pisum sativum* plants [84]. Gapinska *et al.* [65] reported that 150 mM NaCl stress significantly increased the GPX activity in *L. esculentum* Mill. cv. "Perkoz" roots. Leisinger *et al.* [85] reported the upregulation of a GPX homologous gene (Gpxh gene) in *Chlamydomonas reinhardtii* following oxidative stress.

CATs are tetrameric heme-containing enzymes with the potential to dismutate H_2O_2 directly into H_2O and O_2 and are indispensable for ROS detoxification during stress conditions [86]. CAT is important in the removal of H_2O_2 generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration, and purine catabolism [4]. Unpredictable response of CAT activity under metal stress has been reported by various researchers. Under Cd stress, CAT activity declined in *Glycine max* [87], *Phragmites australis* [88], *C. annuum* [83], and *A. thaliana* [89], whereas its activity increased in *O. sativa* [69], *B. juncea* [71], *T. aestivum* [70], *C. arietinum* [73], and *V. mungo* roots [72]. Hsu and Kao [80] reported that pretreatment of rice seedlings with H_2O_2 under nonheat shock conditions resulted in an increase in CAT activity and protected rice seedlings from subsequent Cd stress. It has been reported that a significant increase in CAT activity was observed in

C. arietinum leaves [90] and roots [64] under salinity stress. Srivastava *et al.* [81] reported a decrease in CAT activity in *A. doliolum* under NaCl and Cu^{2+} stress. Simova-Stoilova *et al.* [91] reported increased CAT activity in wheat under drought stress. However, Sharma and Dubey [76] reported a decrease in CAT activity in rice seedlings following drought stress. Pan *et al.* [66] observed that the combined effects of salt and drought stress decrease CAT activity in *G. uralensis* seedlings. It has also been reported that high light condition increased CAT activity in *P. asperata* under drought stress [82]. UV-B stress also led to a significant increase in CAT activity in *Cassia auriculata* seedlings [92]. The extent of oxidative stress in a cell is determined by the amounts of superoxide, H_2O_2, and hydroxyl radicals. Therefore, the balance of SOD, APX, and CAT activities will be crucial for suppressing toxic ROS levels in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms. For example, when CAT activity was reduced in plants, scavenging enzymes such as APX and GPX were upregulated. Unexpected effects can also occur [10]. Compared to plants with suppressed CAT, plants lacking both APX and CAT were less sensitive to oxidative stress [93]. Because photosynthetic activity of these plants was decreased, reduction in APX and CAT might result in suppression of ROS production via chloroplasts [10].

MDHAR associated with APX is also located in peroxisomes and mitochondria, where it scavenges H_2O_2 [46]. Schützendübel *et al.* [94] observed enhanced MDHAR activity in Cd-exposed *Pinus sylvestris* and a declined MDHAR activity in Cd-exposed poplar hybrids (*Populus* × *Canescens*). Sharma and Dubey [76] reported that the activities of enzymes involved in regeneration of ASH, that is, MDHAR, DHAR, and GR, were higher in drought-stressed rice seedlings. It has also been reported that the increase in MDAR activity contributes toward chilling tolerance in tomato fruit [95]. DHAR regenerates ASH from the oxidized state and regulates the cellular ASH redox state, which is crucial for tolerance to various abiotic stresses, leading to the production of ROS [4]. It has also been found that DHAR overexpression also enhances plant tolerance against various abiotic stresses. Plants overexpressing DHAR showed tolerance to Al stress by maintaining high ASH level [96]. Overexpression of DHAR in tobacco protected the plants against ozone toxicity [97], increased salt tolerance in *Arabidopsis* [98], and drought and ozone stress tolerance in tobacco [99]. Guaiacol peroxidase (GPOX) decomposes indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and defense against biotic stresses by scavenging H_2O_2 [4]. GPOX prefers aromatic electron donors such as guaiacol, and pyrogallol usually oxidizes ascorbate at the rate of around 1% that of guaiacol [100]. The activity of GPOX varies considerably depending on plant species and stress conditions. It increased in Cd-exposed plants of *T. aestivum* [101], *A. thaliana* [89], and *C. demersum* [79]. Radotic *et al.* [102] found an initial increase in GPOX activity in spruce needles subjected to Cd stress; however, subsequent Cd treatments caused a decline in the activity. A concomitant increase in GPOX activity in both leaf and root tissues of *Vigna radiate* [103] and *O. sativa* [104] has also been reported under salinity stress.

GR, a flavoprotein oxidoreductase, is a potential enzyme of the ASH–GSH cycle, which plays an essential role in defense system against ROS by sustaining the

reduced status of GSH [105]. GR and GSH play a crucial role in determining the tolerance of a plant under various stresses [106]. GR activity was found increased in the presence of Cd in *C. annuum* [83], *A. thaliana* [68], *V. mungo* [72], *T. aestivum* [70], and *B. juncea* [71]. It has been reported that increased GR activity was observed in the leaf tissues of *C. arietinum* L. cv. Gokce [90] and roots [64] under salt stress. Srivastava *et al.* [81] reported a decline in GR activity in *A. doliolum* under Cu^{2+} stress, but it increased under salt stress. Sharma and Dubey [76] found a significant increase in GR activity in drought-stressed *O. sativa* seedlings. Under high light condition, drought increased the GR activity in *P. asperata* Mast. seedlings, but no prominent drought-induced differences in GR activities were observed in low light seedlings [82]. Plant GSTs are known to have roles in herbicide detoxification, hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification, regulation of apoptosis, and responses to biotic and abiotic stresses [107]. Noctor *et al.* [108] reported that GSTs have the potential to remove cytotoxic or genotoxic compounds, which can react or damage the DNA, RNA, and proteins. In fact, GSTs can reduce peroxides with the help of GSH and produce scavengers of cytotoxic and genotoxic compounds. Increased GST activity was reported in leaves and roots of Cd-exposed *P. sativum* plants [84] and in roots of *O. sativa* and *P. australis* plants [88, 109]. Gapinska *et al.* [65] observed an increased GST activity in *L. esculentum* roots under salinity stress. Drought-tolerant sorghum cv. M35-1 and drought-sensitive cv. SPV-839 were studied for their salinity tolerance to find that cv. M35-1 exhibited efficient H_2O_2 scavenging mechanisms with significantly higher activities of GST and CAT [110].

3.3.2
Nonenzymatic Antioxidants

Ascorbic acid is the most abundant, powerful, and water-soluble antioxidant that prevents or minimizes the damage caused by ROS in plants [111, 112]. ASH is considered as a most powerful ROS scavenger because of its ability to donate electrons in a number of enzymatic and nonenzymatic reactions. It can provide protection to membranes by directly scavenging the $O2^{\bullet -}$ and $OH^{\bullet}$ and by regenerating α-tocopherol from tocopheroxyl radical [4]. Mutants with decreased ascorbic acid levels [113] or altered glutathione content [114] are hypersensitive to stress. While GSH is oxidized by ROS forming GSSG, ascorbate is oxidized to MDA and DHA. Through the ascorbate–glutathione cycle, GSSG, MDA, and DHA can be reduced reforming GSH and ascorbate [10]. Plants increase the activity of GSH biosynthetic enzymes and GSH levels in response to chilling, heat shock, pathogen attack, and drought stress [108, 115, 116]. Demirevska-Kepova *et al.* [117] reported that oxidized ascorbate content was increased during Cd exposure in *H. vulgare* plants. Yang *et al.* [82] observed a significant increase in ASH content in *P. asperata* seedlings under high light condition and drought condition. Agarwal [92] reported that ASH and DHA content, as well as the GSH/GSSG content, was significantly increased by the UV-B stress in *C. auriculata* seedlings. Conversely, a decrease in ASH content in the roots and nodules of *G. max* under Cd stress was also

observed [87]. Cd also decreased the ASH content in *Cucumis sativus* chloroplast and in the leaves of *A. thaliana* and *P. sativum* [118–120], whereas it remained unaffected in *Populus* × *Canescens* roots [119, 120].

GSH is necessary to maintain the normal reduced state of cells so as to counteract the inhibitory effects of ROS-induced oxidative stress [121]. It is a potential scavenger of $^1O_2$, H_2O_2 [122, 123], and most dangerous ROS such as $OH^{\bullet}$ [124]. The role of GSH in the antioxidant defense system provides a strong basis for its use as a stress marker. However, the concentration of cellular GSH has a major effect on its antioxidant function and it varies considerably under abiotic stresses. Furthermore, strong evidence has indicated that an elevated GSH concentration is correlated with the ability of plants to withstand metal-induced oxidative stress. Increased concentration of GSH has been observed with the increasing Cd concentration in *P. sativum* [125], *Sedum alfredii* [126], and *V. mungo* [127]. Srivastava *et al.* [81] reported an appreciable decline in GR activity and GSH pool under Cu stress and a significantly higher increase under salt stress. A high ratio of reduced to oxidized ascorbate and GSH is essential for ROS scavenging in cells. Reduced states of the antioxidants are maintained by GR, MDAR, and DHAR using NADPH as reducing power [128, 129]. In addition, the overall balance among different antioxidants must be tightly controlled. The importance of this balance is evident when cells with enhanced glutathione biosynthesis in chloroplasts show oxidative stress damage, possibly due to changes in the overall redox state of chloroplasts [114].

Tocopherols are considered as a major antioxidant in biomembranes, where they play both antioxidant and nonantioxidant functions and potential scavengers of ROS and lipid radicals [130]. Tocopherols are considered general antioxidants for protection of membrane stability, including quenching or scavenging ROS such as $^1O_2$. Tocopherols have been shown to prevent the chain propagation step in lipid autooxidation, which makes it an effective free radical trap. In addition, it has been estimated that one molecule of α-tocopherol can scavenge up to 120 $^1O_2$ molecules by resonance energy transfer [131]. Recently, it has been found that oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants [132]. Increased levels of α-tocopherol and ASH have been found in tomato following trizole treatment that may help in protecting membranes from oxidative damage, and thus chilling tolerance in tomato plants [133]. Increase in tocopherol during water stress in plants has also been reported by many workers [132, 133]. Srivastava *et al.* [81] reported a general induction in α-tocopherol content in *A. doliolum* under NaCl and Cu^{2+} stress.

3.4 Transgenic Approach in ROS Toxicity in Plants

To combat ROS toxicity and overcome its damaging effects on plant cells, specific roles for antioxidant enzymes have been explored via transgenic approaches. Unlike other organisms, plants possess multiple genes encoding antioxidant enzymes. Different isoforms are specifically targeted to chloroplasts, mitochondria,

peroxisomes, as well as to the cytosol and apoplast [128]. There have been numerous studies on development of abiotic stress-tolerant transgenic plants overexpressing various antioxidant enzymes (Table 3.1). Transgenic rice plants overexpressing *OsMT1a* demonstrated an increased CAT activity and thus an enhanced drought tolerance [134]. Protoplasts with Mn-SOD overexpression showed less oxidative damage, higher H_2O_2 content, and a significant increase in SOD and GR activities under photooxidative stress [135]. Overexpression of a Mn-SOD in transgenic *Arabidopsis* plants also showed increased salt tolerance [136]. Cu/Zn-SOD overexpressing transgenic tobacco plants showed multiple stress tolerance [137], and transgenic *O. sativa* Pusa Basmati-1 were more tolerant to MV-mediated oxidative stress, salinity stress, and drought stress [138]. Overexpression of Mn-SOD in transformed *L. esculentum* plants also showed enhanced tolerance against salt stress [139]. In a study, the maize Cu/Zn-SOD and CAT genes were targeted to the chloroplasts of *Brassica campestris* L. ssp. pekinensis cv. Tropical Pride, and it was reported that exposure of SOD + CAT *B. campestris* plants to 400 ppb SO_2 showed enhanced tolerance than wild type [140]. Furthermore, it was also reported that enhancement of SOD or CAT activity individually had only a minor effect on 400 ng ml^{-1} SO_2 tolerance in *B. campestris* transformed with *E. coli* SOD and CAT genes. It was suggested that the cotransformed strains that overexpressed both SOD and CAT showed high resistance to SO_2 [141]. CAT overexpressed *O. sativa* L. cv. Yuukara or Matsumae showed low-temperature stress tolerance due to effective detoxification of H_2O_2 by CAT [142]. *Nicotiana tabacum* plants transformed with a CAT3 gene from *B. juncea* possessed enhanced tolerance to Cd toxicity and showed better seedling growth and longer roots [143].

Overexpression of APX in *N. tabacum* chloroplasts enhanced plant tolerance to salt and water deficit [137]. Yang *et al.* [134] correlated the enhanced tolerance of *OsMT1a* overexpessing transgenic rice plants to drought stress with an increase in APX activity. Transgenic *A. thaliana* plants with APX1 gene from *H. vulgare* L. exhibited enhanced salt tolerance due to higher APX, SOD, CAT, and GR and lower H_2O_2 and MDA content [144]. Transformed *N. tabacum* plants, which possess *Ipomoea batata* swpa4 gene, displayed improved tolerance to various stresses such as MV, H_2O_2, NaCl, and mannitol and better resisitance to *P. parasitica nicotianae*. swpa4 function as a positive defense signal in the H_2O_2-regulated stress response, and transgenic plants showed 50-fold higher POD specific activity [145]. Transgenic wheat plant overexpressing *E. coli* GR gene showed higher GSH content and GSH/GSH + GSSG ratio than control, but no increase in SOD and GR activities [135]. Overexpression of GR in transgenic *Gossypium hirsutum* L. cv. Coker 312 enhanced chilling stress tolerance and photoprotection [146]. MDAR1 expressing transgenic *N. tabacum* lines exhibited greater tolerance to ozone, salt, and PEG stress owing to higher MDAR activity and higher level of reduced AsA [147]. Transgenic tobacco plants overexpressing DHAR demonstrated better drought and salt tolerance with higher DHAR activity and reduced AsA content [147]. DHAR expressing *A. thaliana* L. (ecotype Wassilewskija) transgenic lines showed better salt tolerance due to a slight increase in DHAR activity and total ascorbate content [97]. Overexpression of GST in transgenic *O. sativa* cv. Zhonghua No.11 improved tolerance to salt and paraquat

Table 3.1 Some instances of ROS scavenging by antioxidant-overexpressing transgenic plants to various abiotic stresses.

Gene/source	Transgenic	Stress tolerance	Reference
Cu/Zn-SOD/*Avicennia marina*	*O. sativa*	Transgenic plants were more tolerant to MV-mediated oxidative stress, salinity stress, and drought stress	[138]
Mn-SOD/*N. plumbaginifolia*	*T. aestivum*	Photooxidative stress tolerance, lower oxidative damage, higher H_2O_2, and significant increase in SOD and GR activities	[135]
CAT/*T. aestivum*	*O. sativa*	Low-temperature stress tolerance due to effective detoxification of H_2O_2 by CAT	[142]
CAT3/*Brassica juncea*	*N. tabacum*	Cd stress tolerance, better seedling growth, and longer roots	[143]
APX1/*H. vulgare*	*A. thaliana*	Salt tolerance due to higher APX, SOD, CAT, and GR and low H_2O_2 and MDA content	[144]
swpa4/*I. batatas*	*N. tabacum*	Resistance to various stresses such as MV, H_2O_2, NaCl, mannitol, and *P. parasitica nicotianae*; swpa4 functions as a positive defense signal in the H_2O_2-regulated stress response and transgenic plants showed 50-fold higher POD specific activity	[145]
GR/*Escherichia coli*	*T. aestivum*	Higher GSH content and GSH/GSH + GSSG ratio than control, no increase in SOD and GR activities	[135]
GR/*A. thaliana*	*G. hirsutum*	Chilling stress tolerance and photoprotection	[146]
MDAR1/*A. thaliana*	*N. tabacum*	Ozone, salt, and PEG stress tolerance due to higher MDAR activity and higher level of reduced AsA	[147]
DHAR/*A. thaliana*	*N. tabacum*	Drought and salt tolerance with higher DHAR activity and reduced AsA content	[147]
DHAR/*O. sativa*	*A. thaliana*	Salt tolerance due to slight increase in DHAR activity and total ascorbate	[147]
GST/*Suaeda salsa*	*O. sativa*	Salt and paraquat stress tolerance due to GST, CAT, and SOD activities	[148]
GST + GPX/*N. tabacum*	*N. tabacum*	Increased thermal or salt stress tolerance due to glutathione and ascorbate content	[110]
GPX/*Chlamydomonas*	*N. tabacum*	Tolerant to MV under moderate light intensity, chilling stress under high light intensity, or salt stress due to low MDA and high photosynthesis and antioxidative system	[149]
GPX-2/*Synechocystis* PCC 6803	*A. thaliana*	Tolerance to H_2O_2, Fe ions, MV, chilling, high salinity, or drought stresses	[150]

stress due to GST, CAT, and SOD activity [148]. GST + GPX expressing transgenic tobacco exhibited increased thermal or salt stress tolerance due to glutathione and ascorbate content [110]. Transgenic tobacco plants that overexpressed GPX demonstrated better tolerance to MV under moderate light intensity, chilling stress under high light intensity, or salt stress due to low MDA and high photosynthesis and antioxidative system [149]. GPX-2 expressing *Arabidopsis* transgenic lines showed enhanced tolerance to H_2O_2, Fe ions, MV, chilling, high salinity, or drought stresses [150].

3.5 Conclusions

ROS, which are partially reduced or activated derivatives of oxygen ($^1O_2$, $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, and $HO_2^{\bullet -}$) invariably generated as spin-off by plants during various metabolic processes in different cellular compartments such as chloroplast, mitochondria, peroxisomes, cytosol, plasma membrane, and apoplastic space, are highly reactive and toxic and can lead to the oxidative destruction of cells (oxidative stress). ROS can react with cellular components (lipids, proteins, and nucleic acids) and cause lipid peroxidation, protein oxidation, DNA damage, membrane damage, and inactivation of enzymes, thus affecting various physiological and biochemical processes as well as cell viability. Plants have evolved a complex array of ROS scavenging mechanisms to retain low ROS level, and thereby the detrimental effects of excessively high ROS concentrations could be avoided. Enzymatic antioxidant defense systems of plants consist of a variety of ROS scavengers, such as SOD, APX, GPX, CAT, MDHAR, DHAR, GPOX, GR, and GST, and nonenzymatic low molecular metabolites, such as ASH, GSH, α-tocopherol, proline, carotenoids, and flavonoids. However, numerous biotic and abiotic stresses such as plant diseases, drought, salinity, extreme temperatures, excess light, pollutants, xenobiotics, ultraviolet radiation, ozone, hypoxia, nutrient deficiency, and so on upset the equilibrium between ROS generation and scavenging, leading to overproduction and higher accumulation of ROS and eventually oxidative stress. High accumulation of ROS in plant cells affects various cellular functions through damaging nucleic acids, protein oxidation, and lipid peroxidation, which eventually results in cell death. ROS toxicity resulting from various biotic and abiotic stress factors is considered to be one of the major causes of low crop productivity worldwide. However, they cannot be eliminated completely because plants use ROS as second messengers in signal transduction cascades in diverse physiological processes. It has become evident that ROS also play an important signaling role in plants controlling processes such as growth, development, response to biotic and abiotic stresses, and programmed cell death. This suggests a dual role for ROS as both toxic by-products of aerobic metabolism and key regulators of growth, development, and defense pathways. It is still not clearly understood how this dual role is controlled in plants; however, it is quite clear that the steady-state level of ROS in cells needs to be tightly regulated. Further genomics, proteomics, and metabolomics studies, combined with newly emerging technologies, may provide an insight

into the networks involved in different ROS-related plant processes. A thorough and complete understanding of the ROS gene network may lead to the identification of genes, which can be exploited to modulate/transform ROS-related metabolisms in plant for the development of better performing transgenic crop plants against biotic and abiotic stress.

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References

1 Lichtenthaler, H.K. (1996) Vegetation stress: an introduction to the stress concept in plants. *Plant Physiol.*, **148**, 4–14.
2 Li, Z.R., Wakao, S., Fischer, B.B., and Niyogi, K.K. (2009) Sensing and responding to excess light. *Annu. Rev. Plant Biol.*, **60**, 239–260.
3 Navrot, N., Rouhier, N., Gelhaye, E., and Jacquot, J.P. (2007) Reactive oxygen species generation and antioxidant systems in plant mitochondria. *Physiol. Plant.*, **129**, 185–195.
4 Gill, S.S. and Tuteja, N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.*, **48**, 909–930.
5 Møller, I.M., Jensen, P.E., and Hansson, A. (2007) Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol.*, **58**, 459–481.
6 Rinalducci, S., Murgiano, L., and Zolla, L. (2008) Redox proteomics: basic principles and future perspectives for the detection of protein oxidation in plants. *J. Exp. Bot.*, **59**, 3781–3801.
7 Foyer, C.H. and Noctor, G. (2005) Redox homeostatis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell*, **17**, 1866–1875.
8 Bhattacharjee, S. (2005) Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plant. *Curr. Sci.*, **89**, 1113–1121.
9 Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.*, **7**, 405–410.
10 Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.*, **55**, 373–399.
11 Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.*, **444**, 139–158.
12 Tuteja, N. (2007) Mechanisms of high salinity tolerance in plants. *Methods Enzymol.*, **428**, 419–438.
13 Tuteja, N. (2010) Cold, salt and drought stress, in *Plant Stress Biology: From Genomics towards System Biology* (ed. H. Hirt), Wiley-Blackwell, Weinheim, pp. 137–159.
14 Khan, N.A. and Singh, S. (eds) (2008) *Abiotic Stress and Plant Responses*, IK International, New Delhi.
15 Gill, S.S., Khan, N.A., Anjum, N.A., and Tuteja, N. (2011) Amelioration of cadmium stress in crop plants by nutrients management: morphological, physiological and biochemical aspects. *Plant Stress*, **5**, 1–23.

16 Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E., and Schroeder, J.I. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature*, **406**, 731–734.

17 Gechev, T.S. and Hille, J. (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. *J. Cell Biol.*, **168**, 17–20.

18 Laloi, C., Stachowiak, M., Pers-Kamczyc, E., Warzych, E., Murgia, I., and Apel, K. (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, **104**, 672–677.

19 Miller, G., Suzuki, N., Rizhsky, L., Hegie, A., Koussevitzky, S., and Mittler, R. (2007) Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. *Plant Physiol.*, **144**, 1777–1785.

20 Shaw, S.L. and Long, S.R. (2003) Nod factor inhibition of reactive oxygen efflux in a host legume. *Plant Physiol.*, **132**, 2196–2204.

21 Rubio, M.C., James, E.K., Clemente, M.R., Bucciarelli, B., Fedorova, M., Vance, C.P., and Becana, M. (2004) Localization of superoxide dismutases and hydrogen peroxide in legume root nodules. *Mol. Plant Microbe Interact.*, **17**, 1294–1305.

22 Baptista, P., Martins, A., Pais, M.S., Tavares, R.M., and Lino-Neto, T. (2007) Involvement of reactive oxygen species during early stages of ectomycorrhiza establishment between *Castanea sativa* and *Pisolithus tinctorius*. *Mycorrhiza*, **17**, 185–193.

23 Bailey-Serres, J. and Mittler, R. (2006) The roles of reactive oxygen species in plant cells. *Plant Physiol.*, **141**, 311.

24 Gratao, P.L., Polle, A., Lea, P.J., and Azevedo, R.A. (2005) Making the life of heavy metal stressed plants a little easier. *Funct. Plant Biol.*, **32**, 481–494.

25 Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004) Reactive oxygen gene network of plants. *Trends Plant Sci.*, **9**, 490–498.

26 Chen, C. and Dickman, M.B. (2005) Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*. *Proc. Natl. Acad. Sci. USA*, **102**, 3459–3464.

27 Dalton, T.P., Shertzer, H.G., and Puga, A. (1999) Regulation of gene expression by reactive oxygen. *Annu. Rev. Pharmacol. Toxicol.*, **39**, 67–101.

28 Foyer, C.H. and Noctor, G. (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxid. Redox Signal.*, **11**, 861–905.

29 Takahashi, S. and Murata, N. (2008) How do environmental stresses accelerate photoinhibition? *Trends Plant Sci.*, **13**, 178–182.

30 Wise, R.R. and Naylor, A.W. (1987) Chilling-enhanced photooxidation: evidence for the role of singlet oxygen and superoxide in the breakdown of pigments and endogenous antioxidants. *Plant Physiol.*, **83**, 278–282.

31 Elstner, E.F. (1991) Mechanism of oxygen activation in different compartments, in *Active Oxygen/Oxidative Stress and Plant Metabolism* (eds E.J. Pell and K.L. Steffen), American Society of Plant Physiologists, Roseville, pp. 13–25.

32 Takahashi, M. and Asada, K. (1988) Superoxide production in aprotic interior of chloroplast thylakoids. *Arch. Biochem. Biophys.*, **267**, 714–722.

33 Buchert, F. and Forreiter, C. (2010) Singlet oxygen inhibits ATPase and proton translocation activity of the thylakoid ATP synthase CF1CFo. *FEBS Lett.*, **584**, 147–152.

34 Mur, L.A.J., Kenton, P., Lloyd, A.J., Ougham, H., and Prats, E. (2008) The hypersensitive response: the centenary is upon us but how much do we know? *J. Exp. Bot.*, **59**, 501–520.

35 Gray, J., Janick-Buckner, D., Buckner, B., Close, P.S., and Johal, G.S. (2002) Light-dependent death of maize lls1 cells is mediated by mature chloroplasts. *Plant Physiol.*, **130**, 1894–1907.

36 Chen, S. and Dickman, M.B. (2004) Bcl-2 family members localize to tobacco

chloroplasts and inhibit programmed cell death induced by chloroplast-targeted herbicides. *J. Exp. Bot.*, **55**, 2617–2623.
37 Doyle, S.M., Diamond, M., and McCabe, P.F. (2010) Chloroplast and reactive oxygen species involvement in apoptotic-like programmed cell death in *Arabidopsis* suspension cultures. *J. Exp. Bot.*, **61**, 473–482.
38 Taylor, N.L., Tan, Y.F., Jacoby, R.P., and Millar, A.H. (2009) Abiotic environmental stress induced changes in the *Arabidopsis thaliana* chloroplast, mitochondria and peroxisome proteomes. *J. Proteomics*, **72**, 367–378.
39 Rhoads, D.M., Umbach, A.L., Subbaiah, C.C., and Siedow, J.N. (2006) Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. *Plant Physiol.*, **141**, 357–366.
40 Quan, L.J., Zhang, B., Shi, W.W., and Li, H.Y. (2008) Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *J. Integr. Plant Biol.*, **50**, 2–18.
41 Moller, I.M. (2001) Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annu. Rev. Plant Physiol. Mol. Biol.*, **52**, 561–591.
42 Sweetlove, L.J. and Foyer, C.H. (2004) Roles for reactive oxygen species and antioxidants in plant mitochondria, in *Plant Mitochondria: From Genome to Function*, vol. **1**, Advances in Photosynthesis and Respiration (eds D.A. Day, A.H. Millar, and J. Whelan), Kluwer Academic Press, Dordrecht, pp. 307–320.
43 Grene, R. (2002) Oxidative stress and acclimation mechanisms in plants, in *The Arabidopsis Book* (eds C.R. Somerville and E.M. Myerowitz), American Society of Plant Biologists, Rockville, MD, http://www.aspb.org/publications/Arabidopsis.
44 Almeras F E., Stolz, S., Vollenweider, S., Reymond, P., Mene-Saffrane, L., and Farmer, E.E. (2003) Reactive electrophile species activate defense gene expression in Arabidopsis. *Plant J.*, **34**, 205–216.
45 Gao, C., Xing, D., Li, L., and Zhang, L. (2008) Implication of reactive oxygen species and mitochondrial dysfunction in the early stages of plant programmed cell death induced by ultraviolet-C overexposure. *Planta*, **227**, 755–767.
46 del Río, L.A., Corpas, F.J., Sandalio, L.M., Palma, J.M., Gómez, M., and Barroso, J.B. (2002) Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. *J. Exp. Bot.*, **53**, 1255–1272.
47 Corpas, F.J., Barroso, J.B., and del Río, L.A. (2001) Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. *Trends Plant Sci.*, **6**, 145–150.
48 del Rio, L.A., Sandalio, L.M., Corpas, F.J., Palma, J.M., and Barroso, J.B. (2006) Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. *Plant Physiol.*, **141**, 330–335.
49 McDowell, J.M. and Dangl, J.L. (2000) Signal transduction in the plant immune response. *Trends Biochem. Sci.*, **25**, 79–82.
50 Grant, J.J. and Loake, G.J. (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiol.*, **124**, 21–29.
51 Halliwell, B. (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol.*, **141**, 312–322.
52 Jaspers, P. and Kangasjarvi, J. (2010) Reactive oxygen species in abiotic stress signaling. *Physiol. Plant.*, **138**, 405–413.
53 Monshausen, G.B. and Gilroy, S. (2009) Feeling green: mechanosensing in plants. *Trends Cell Biol.*, **19**, 228–235.
54 Torres, M.A. and Dangl, J.L. (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.*, **8**, 397–403.
55 Pourrut, B., Perchet, G., Silvestre, J., Cecchi, M., Guiresse, M., and Pinelli, E. (2008) Potential role of NADPH-oxidase in early steps of lead-induced oxidative burst in *Vicia faba* roots. *J. Plant Physiol.*, **165**, 571–579.
56 Leshem, Y., Seri, L., and Levine, A. (2007) Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt

stress leads to intracellular production of reactive oxygen species and salt tolerance. *Plant J.*, **51**, 185–197.

57 Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M.A., Shulaev, V., Dangl, J.L., and Mittler, R. (2009) The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Sci. Signal.*, **2**, 45.

58 Dybing, E., Nelson, J.R., Mitchell, J.R., Sesame, H.A., and Gillette, J.R. (1976) Oxidation of a methyldopa and other catechols by chytochromes R450-generated superoxide anion: possible mechanism of methyldopa hepatitis. *Mol. Pharmacol.*, **12**, 911–920.

59 Bolwell, G.P. and Woftastek, P. (1997) Mechanism for the generation of reactive oxygen species in plant defense—broad perspective. *Physiol. Mol. Plant Pathol.*, **51**, 347–349.

60 Moschou, P.N., Paschalidis, K.A., Delis, I.D., Andriopoulou, A.H., Lagiotis, G.D., Yakoumakis, D.I., and Roubelakis-Angelakis, K.A. (2008) Spermidine exodus and oxidation in the apoplast induced by abiotic stress is responsible for H_2O_2 signatures that direct tolerance responses in tobacco. *Plant Cell*, **20**, 1708–1724.

61 Rucińska-Sobkowiak, R. (2010) Oxidative stress in plants exposed to heavy metals. *Postepy Biochem.*, **56**, 191–200.

62 Szymańska, R. and Strzałka, K. (2010) Reactive oxygen species in plants—production, deactivation and role in signal transduction. *Postepy Biochem.*, **56**, 182–190.

63 Harinasut, P., Poonsopa, D., Roengmongkol, K., and Charoensataporn, R. (2003) Salinity effects on antioxidant enzymes in mulberry cultivar. *Sci. Asia*, **29**, 109–113.

64 Kukreja, S., Nandval, A.S., Kumar, N., Sharma, S.K., Sharma, S.K., Unvi, V., and Sharma, P.K. (2005) Plant water status, H_2O_2 scavenging enzymes, ethylene evolution and membrane integrity of *Cicer arietinum* roots as affected by salinity. *Biol. Plant.*, **49**, 305–308.

65 Gapinska, M., Sklodowska, M., and Gabara, B. (2008) Effect of short- and long-term salinity on the activities of antioxidative enzymes and lipid peroxidation in tomato roots. *Acta Physiol. Plant.*, **30**, 11–18.

66 Pan, Y., Wu, L.J., and Yu, Z.L. (2006) Effect of salt and drought stress on antioxidant enzymes activities and SOD isoenzymes of liquorice (*Glycyrrhiza uralensis* Fisch). *Plant Growth Regul.*, **49**, 157–165.

67 Guo, T., Zhang, G., Zhou, M., Wu, F., and Chen, J. (2004) Effects of aluminum and cadmium toxicity on growth and antioxidant enzyme activities of two barley genotypes with different Al resistance. *Plant Soil*, **258**, 241–248.

68 Skorzynska-Polit, E., Drazkiewicz, M., and Krupa, Z. (2003/04) The activity of the antioxidative system in cadmium-treated *Arabidopsis thaliana*. *Biol. Plant.*, **47**, 71–78.

69 Hsu, Y.T. and Kao, C.H. (2004) Cadmium toxicity is reduced by nitric oxide in rice leaves. *Plant Growth Regul.*, **42**, 227–238.

70 Khan, N.A., Samiullah, Singh, S., and Nazar, R. (2007) Activities of antioxidative enzymes, sulphur assimilation, photosynthetic activity and growth of wheat (*Triticum aestivum*) cultivars differing in yield potential under cadmium stress. *J. Agro. Crop Sci.*, **193**, 435–444.

71 Mobin, M. and Khan, N.A. (2007) Photosynthetic activity, pigment composition and antioxidative response of two mustard (*Brassica juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress. *J. Plant Physiol.*, **164**, 601–610.

72 Singh, S., Khan, N.A., Nazar, R., and Anjum, N.A. (2008) Photosynthetic traits and activities of antioxidant enzymes in blackgram (*Vigna mungo* L. Hepper) under cadmium stress. *Am. J. Plant Physiol.*, **3**, 25–32.

73 Hasan, S.A., Hayat, S., Ali, B., and Ahmad, A. (2008) 28-Homobrassinolide protects chickpea (*Cicer arietinum*) from cadmium toxicity by stimulating antioxidants. *Environ. Pollut.*, **151**, 60–66.

74 Zlatev, Z.S., Lidon, F.C., Ramalho, J.C., and Yordanov, I.T. (2006) Comparison of

resistance to drought of three bean cultivars. *Biol. Plant.*, **50**, 389–394.

75 Wang, L., Zhou, Q., Ding, L., and Sun, Y. (2008) Effect of cadmium toxicity on nitrogen metabolism in leaves of *Solanum nigrum* L. as a newly found cadmium hyperaccumulator. *J. Hazard. Mater.*, **154**, 818–825.

76 Sharma, P. and Dubey, R.S. (2005) Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: role of osmolytes as enzyme protectant. *J. Plant Physiol.*, **162**, 854–864.

77 Wang, C.A. and Li, R.C. (2008) Enhancement of superoxide dismutase activity in the leaves of white clover (*Trifolium repens* L.) in response to polyethylene glycol-induced water stress. *Acta Physiol. Plant.*, **30**, 841–847.

78 Simonovicova, M., Tamás, L., Huttová, J., and Mistrík, I. (2004) Effect of aluminium on oxidative stress related enzymes activities in barley roots. *Biol. Plant.*, **48**, 261–266.

79 Arvind, P. and Prasad, M.N.V. (2003) Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* L: a free-floating freshwater macrophyte. *Plant Physiol. Biochem.*, **41**, 391–397.

80 Hsu, Y.T. and Kao, C.H. (2007) Heat shock-mediated H_2O_2 accumulation and protection against Cd toxicity in rice seedlings. *Plant Soil*, **300**, 137–147.

81 Srivastava, A.K., Bhargava, P., and Rai, L.C. (2005) Salinity and copper-induced oxidative damage and changes in antioxidative defense system of *Anabaena doliolum*. *World J. Microbiol. Biotechnol.*, **22**, 1291–1298.

82 Yang, Y., Han, C., Liu, Q., Lin, B., and Wang, J.W. (2008) Effect of drought and low light on growth and enzymatic antioxidant system of *Picea asperata* seedlings. *Acta Physiol. Plant.*, **30**, 433–440.

83 Leon, A.M., Palma, J.M., Corpas, F.J., Gomez, M., Romero-Puertas, M.C., Chatterjee, D., Mateos, R.M., del Rio, L.A., and Sandalio, L.M. (2002) Antioxidant enzymes in cultivars of pepper plants with different sensitivity to cadmium. *Plant Physiol. Biochem.*, **40**, 813–820.

84 Dixit, V., Pandey, V., and Shyam, R. (2001) Differential oxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L cv. Azad). *J. Exp. Bot.*, **52**, 1101–1109.

85 Leisinger, U., Rüfenacht, K., Fischer, B., Pesaro, M., Spengler, A., Zehnder, A.J.B., and Eggen, R.I.L. (2001) The glutathione peroxidase homologous gene from *Chlamydomonas reinhardtii* is transcriptionally up-regulated by singlet oxygen. *Plant Mol. Biol.*, **46**, 395–408.

86 Garg, N. and Manchanda, G. (2009) ROS generation in plants: boon or bane? *Plant Biosyst.*, **143**, 8–96.

87 Balestrasse, K.B., Gardey, L., Gallego, S.M., and Tomaro, M.L. (2001) Response of antioxidant defence system in soybean nodules and roots subjected to cadmium stress. *Aust. J. Plant Physiol.*, **28**, 497–504.

88 Iannelli, M.A., Pietrini, F., Fiore, L., Petrilli, L., and Massacci, A. (2002) Antioxidant response to cadmium in *Phragmites australis* plants. *Plant Physiol. Biochem.*, **40**, 977–982.

89 Cho, U. and Seo, N. (2005) Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. *Plant Sci.*, **168**, 113–120.

90 Eyidogan, F. and Oz, M.T. (2005) Effect of salinity on antioxidant responses of chickpea seedlings. *Acta Physiol. Plant.*, **29**, 485–493.

91 Simova-Stoilova, L., Vaseva, I., Grigorova, B., Demirevska, K., and Feller, U. (2010) Proteolytic activity and cysteine protease expression in wheat leaves under severe soil drought and recovery. *Plant Physiol. Biochem.*, **48**, 200–206.

92 Agarwal, S. (2007) Increased antioxidant activity in *Cassia* seedlings under UV-B radiation. *Biol. Plant.*, **51**, 157–160.

93 Rizhsky, L., Hallak-Herr, E., Van Breusegem, F., Rachmilevitch, S., Barr, J.E., *et al.* (2002) Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants

lacking ascorbate peroxidase or catalase. *Plant J.*, **32**, 329–342.

94 Schützendübel, A., Schwanz, P., Teichmann, T., Gross, K., Langenfeld-Heyser, R., Godbold, D.L., and Polle, A. (2001) Cadmium-induced changes in antioxidative systems, H_2O_2 content and differentiation in pine (*Pinus sylvestris*) roots. *Plant Physiol.*, **127**, 887–898.

95 Stevens, R., Page, D., Gouble, B., Garchery, C., Zamir, D., and Causse, M. (2008) Tomato fruit ascorbic acid content is linked with monodehydroascorbate reductase activity and tolerance to chilling stress. *Plant Cell Environ.*, **31**, 1086–1096.

96 Yin, L., Wang, S., Eltayeb, A.E., Uddin, M.I., Yamamoto, Y., Tsuji, W., Takeuchi, Y., and Tanaka, K. (2010) Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminium stress in transgenic tobacco. *Planta*, **231**, 609–621.

97 Chen, Z. and Gallie, D.R. (2005) Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. *Plant Physiol.*, **138**, 1673–1689.

98 Ushimaru, U., Nakagawa, T., Fujioka, Y., Daicho, K., Naito, M., Yamauchi, Y., Nonaka, H., Amako, K., Yamawaki, K., and Murata, N. (2006) Transgenic *Arabidopsis* plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. *J. Plant Physiol.*, **163**, 1179–1184.

99 Eltayeb, A.E., Kawano, N., Badawi, G.H., Kaminaka, H., Sanekata, T., Morishima, I., Shibahara, T., Inanaga, S., and Tanaka, K. (2006) Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. *Physiol. Plant.*, **127**, 57–65.

100 Asada, K. (1999) The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 601–639.

101 Milone, M.T., Sgherri, C., Clijters, H., and Navari-Izzo, F. (2003) Antioxidative responses of wheat treated with realistic concentrations of cadmium. *Environ. Exp. Bot.*, **50**, 265–273.

102 Radotic, K., Ducic, T., and Mutavdzic, D. (2000) Changes in peroxidase activity and isoenzymes in spruce needles after exposure to different concentrations of cadmium. *Environ. Exp. Bot.*, **44**, 105–113.

103 Panda, S.K. (2001) Response of green gram seeds under salinity stress. *Indian J. Plant Physiol.*, **6**, 438–440.

104 Koji, Y., Shiro, M., Michio, K., Mitsutaka, T., and Hiroshi, M. (2009) Antioxidant capacity and damages caused by salinity stress in apical and basal regions of rice leaf. *Plant Prod. Sci.*, **12**, 319–326.

105 Edwards, E.A., Rawsthorne, S., and Mullineaux, P.M. (1990) Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (*Pisum sativum* L.). *Planta*, **180**, 278–284.

106 Chalapathi Rao, A.S.V. and Reddy, A.R. (2008) Glutathione reductase: a putative redox regulatory system in plant cells, in *Sulfur Assimilation and Abiotic Stresses in Plants* (eds N.A. Khan, S. Singh, and S. Umar), Springer, Berlin, pp. 111–147.

107 Dixon, D.P., Skipsey, M., and Edwards, R. (2010) Roles for glutathione transferases in plant secondary metabolism. *Phytochemistry*, **71**, 338–350.

108 Noctor, G., Gomez, L., Vanacker, H., and Foyer, C.H. (2002) Interactions between biosynthesis, compartmentation, and transport in the control of glutathione homeostasis and signalling. *J. Exp. Bot.*, **53**, 1283–1304.

109 Moons, A. (2003) Osgstu3 and osgtu4, encoding tau class glutathione S-transferases, are heavy metal- and hypoxic stress-induced and differentially salt stress responsive in rice roots. *FEBS Lett.*, **553**, 427–432.

110 Jogeswar, G., Pallela, R., Jakka, N.M., Reddy, P.S., Venkateswara Rao, J., Sreenivasulu, N., and Kavi Kishor, P.B. (2006) Antioxidative response in different sorghum species under short-term

salinity stress. *Acta Physiol. Plant.*, **28**, 465–475.

111 Smirnoff, N. (2005) Ascorbate, tocopherol and carotenoids: metabolism, pathway engineering and functions, in *Antioxidants and Reactive Oxygen Species in Plants* (ed. N. Smirnoff), Blackwell Publishing Ltd., Oxford, pp. 53–86.

112 Athar, H.R., Khan, A., and Ashraf, M. (2008) Exogenously applied ascorbic acid alleviates salt-induced oxidative stress in wheat. *Environ. Exp. Bot.*, **63**, 224–231.

113 Conklin, P.L., Williams, E.H., and Last, R.L. (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. *Proc. Natl. Acad. Sci. USA*, **93**, 9970–9974.

114 Creissen, G., Firmin, J., Fryer, M., Kular, B., Leyland, N., *et al.* (1999) Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. *Plant Cell*, **11**, 1277–1292.

115 Vanacker, H., Carver, T.L.W., and Foyer, C.H. (2000) Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley–powdery mildew interaction. *Plant Physiol.*, **123**, 1289–1300.

116 Vernoux, T., Sanchez-Fernandez, R., and May, M. (2002) Glutathione biosynthesis in plants, in *Oxidative Stress in Plants* (eds D. Inze and M.V. Montagu), Taylor & Francis, London, pp. 297–311.

117 Demirevska-Kepova, K., Simova-Stoilova, L., Stoyanova, Z.P., and Feller, U. (2006) Cadmium stress in barley: growth, leaf pigment, and protein composition and detoxification of reactive oxygen species. *J. Plant Nutr.*, **29**, 451–468.

118 Zhang, F.Q., Shi, W.Y., Jin, Z.X., and Shen, Z.G. (2003) Response of antioxidative enzymes in cucumber chloroplast to cadmium toxicity. *J. Plant Nutr.*, **26**, 1779–1788.

119 Romero-Puertas, M.C., Corpas, F.J., Rodrıguez-Serrano, M., Gomez, M., del Rıo, L.A., and Sandalio, L.M. (2007) Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. *J. Plant Physiol.*, **164**, 1346–1357.

120 Schützendübel, A., Nikolova, P., Rudolf, C., and Polle, A. (2002) Cadmium and H_2O_2 induced oxidative stress in *Populas* × *canescens* roots. *Plant Physiol. Biochem.*, **40**, 577–584.

121 Meyer, A.J. (2008) The integration of glutathione homeostasis and redox signaling. *J. Plant Physiol.*, **165**, 1390–1403.

122 Noctor, G. and Foyer, C.H. (1998) A re-evaluation of the ATP:NADPH budget during C3 photosynthesis: a contribution from nitrate assimilation and its associated respiratory activity? *J. Exp. Bot.*, **49**, 1895–1908.

123 Briviba, K., Klotz, L.O., and Sies, H. (1997) Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems. *J. Biol. Chem.*, **378**, 1259–1265.

124 Larson, R.A. (1988) The antioxidants of higher plants. *Phytochemistry*, **27**, 969–978.

125 Metwally, A., Safronova, V.I., Belimov, A.A., and Dietz, K.J. (2005) Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. *J. Exp. Bot.*, **56**, 167–178.

126 Sun, Q., Yec, Z.H., Wang, X.R., and Wong, M.H. (2007) Cadmium hyperaccumulation leads to an increase of glutathione rather than phytochelatins in the cadmium hyperaccumulator *Sedum alfredii*. *J. Plant Physiol.*, **164**, 1489–1498.

127 Molina, A.S., Nievas, C., Chaca, M.V.P., Garibotto, F., González, U., Marsá, S.M., Luna, C., Giménez, M.S., and Zirulnik, F. (2008) Cadmium-induced oxidative damage and antioxidative defense mechanisms in *Vigna mungo* L. *Plant Growth Regul.*, **56**, 285–295.

128 Asada, K. and Takahashi, M. (1987) Production and scavenging of active oxygen in photosynthesis, in *Photoinhibition* (eds D.J. Kyle, C.B. Osborne, and C.J. Arntzen), Elsevier, Amsterdam, pp. 227–287.

129 Tsugane, K., Kobayashi, K., Niwa, Y., Ohba, Y., Wada, K., and Kobayashi, H. (1999) A recessive *Arabidopsis* mutant that grows enhanced active oxygen detoxification. *Plant Cell*, **11**, 1195–1206.

130 Hollander-Czytko, H., Grabowski, J., Sandorf, I., Weckermann, K., and Weiler, E.W. (2005) Tocopherol content and activities of tyrosine aminotransferase and cysteine lyase in *Arabidopsis* under stress conditions. *J. Plant Physiol.*, **162**, 767–770.

131 Munné-Bosch, S. (2005) The role of α-tocopherol in plant stress tolerance. *J. Plant Physiol.*, **162**, 743–748.

132 Wu, G., Wei, Z.K., and Shao, H.B. (2007) The mutual responses of higher plants to environment: physiological and microbiological aspects. *Biointerfaces*, **59**, 113–119.

133 Shao, H.B., Chu, L.Y., Wu, G., Zhang, J.H., Lu, Z.H., and Hu, Y.C. (2007) Changes of some antioxidative physiological indices under soil water deficits among 10 wheat (*Triticum aestivum* L.) genotypes at tillering stage. *Colloid. Surf. B Biointerfaces*, **54**, 143–149.

134 Yang, Z., Wu, Y., Li, Y., Ling, H.-Q., and Chu, C. (2009) OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice. *Plant Mol. Biol.*, **70**, 219–229.

135 Melchiorre, M., Robert, G., Trippi, V., Racca, R., and Lascano, H.R. (2009) Superoxide dismutase and glutathione reductase overexpression in wheat protoplast: photooxidative stress tolerance and changes in cellular redox state. *Plant Growth Regul.*, **57**, 57–68.

136 Wang, Y., Ying, Y., Chen, J., and Wang, X.C. (2004) Transgenic *Arabidopsis* overexpressing Mn-SOD enhanced salt-tolerance. *Plant Sci.*, **167**, 671–677.

137 Badawi, G.H., Yamauchi, Y., Shimada, E., Sasaki, R., Kawano, N., and Tanaka, K. (2004) Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (*Nicotiana tabacum*) chloroplasts. *Plant Sci.*, **166**, 919–928.

138 Prashanth, S.R., Sadhasivam, V., and Parida, A. (2008) Over expression of cytosolic copper/zinc superoxide dismutase from a mangrove plant *Avicennia marina* in indica rice var Pusa Basmati-1 confers abiotic stress tolerance. *Transgenic Res.*, **17**, 281–291.

139 Wang, Y., Wisniewski, M., Meilan, R., Uratsu, S.L., Cui, M.G., Dandekar, A., and Fuchigami, L. (2007) Ectopic expression of Mn-SOD in *Lycopersicon esculentum* leads to enhanced tolerance to salt and oxidative stress. *J. Appl. Hort.*, **9**, 3–8.

140 Tseng, M.J., Liu, C.W., and Yiu, J.C. (2007) Enhanced tolerance to sulfur dioxide and salt stress of transgenic Chinese cabbage plants expressing both superoxide dismutase and catalase in chloroplasts. *Plant Physiol. Biochem.*, **45**, 822–833.

141 Tseng, M.J., Liu, C.W., and Yiu, J.C. (2007) Tolerance to sulfur dioxide in transgenic Chinese cabbage transformed with both the superoxide dismutase containing manganese and catalase genes of *Escherichia coli*. *Sci. Hort.*, **115**, 101–110.

142 Matsumura, T., Tabayashi, N., Kamagata, Y., Souma, C., and Saruyama, H. (2002) Wheat catalase expressed in transgenic rice can improve tolerance against low temperature stress. *Physiol. Plant.*, **116**, 317–327.

143 Gichner, T., Patkova, Z., Szakova, J., and Demnerova, K. (2004) Cadmium induces DNA damages in tobacco roots, but no DNA damage, somatic mutations or homologous recombinations in tobacco leaves. *Mutat. Res. Genet. Toxicol. Environ. Mut.*, **559**, 49–57.

144 Xu, W.F., Shi, W.M., Ueda, A., and Takabe, T. (2008) Mechanisms of salt tolerance in transgenic *Arabidopsis thaliana* carrying a peroxisomal ascorbate peroxidase gene from barley. *Pedosphere*, **18**, 486–495.

145 Kim, Y.H., Kim, C.Y., Song, W.K., Park, D.S., Kwon, S.Y., Lee, H.S., Bang, J.W., and Kwak, S.S. (2008) Overexpression of sweetpotato swpa4 peroxidase results in increased hydrogen peroxide production and enhances stress tolerance in tobacco. *Planta*, **227**, 867–881.

146 Kornyeyev, D., Logan, B.A., Payton, P., Allen, R.D., and Holaday, A.S. (2003) Elevated chloroplastic glutathione reductase activities decrease chilling-induced photoinhibition by increasing

rates of photochemistry, but not thermal energy dissipation, in transgenic cotton. *Funct. Plant Biol.*, **30**, 101–110.

147 Eltayeb, A.E., Kawano, N., Badawi, G.H., Kaminaka, H., Sanekata, T., Shibahara, T., Inanaga, S., and Tanaka, K. (2007) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. *Planta*, **225**, 1255–1264.

148 Zhao, F. and Zhang, H. (2006) Salt and paraquat stress tolerance results from coexpression of the *Suaeda salsa* glutathione S-transferase and catalase in transgenic rice. *Plant Cell Tiss. Org. Cult.*, **86**, 349–358.

149 Yoshimura, K., Miyao, K., Gaber, A., Takeda, T., Kanaboshi, H., Miyasaka, H., and Shigeoka, S. (2004) Enhancement of stress tolerance in transgenic tobacco plants overexpressing *Chlamydomonas* glutathione peroxidase in chloroplasts or cytosol. *Plant J.*, **37**, 21–33.

150 Gaber, A., Yoshimura, K., Yamamoto, T., Yabuta, Y., Takeda, T., Miyasaka, H., Nakano, Y., and Shigeoka, S. (2006) Glutathione peroxidase-like protein of *Synechocystis* PCC 6803 confers tolerance to oxidative and environmental stresses in transgenic *Arabidopsis*. *Physiol. Plant.*, **128**, 251–262.

4
Salinity Stress: A Major Constraint in Crop Production

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Salinity is a major constraint in crop production worldwide. Salinity affects nearly 20% of the world's cultivated area and about half the world's total irrigated lands. Saline soil is characterized by the toxic levels of chlorides and sulfates of sodium. The problem of soil salinity is increasing due to irrigation, improper drainage, entry of seawater in coastal areas, and salt accumulation in arid and semiarid regions. Sodium is an essential micronutrient for some of the plants, but most crop plants are natrophobic. Salinity is detrimental to plant growth as it causes nutritional constraints by decreasing uptake of phosphorus, potassium, nitrate, and calcium and leads to ion cytotoxicity and osmotic stress. Under salinity, ions such as Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the function of these proteins. Ionic toxicity, osmotic stress, and nutritional defects under salinity lead to metabolic imbalances and oxidative stress. Plant salt tolerance mechanisms can be grouped into cellular homeostasis (including ion homeostasis and osmotic adjustment), stress damage control (repair and detoxification), and growth regulation. Osmotic stress induces several proteins in vegetative tissues of higher plants, which are related to late-embryogenesis abundant (LEA) proteins and thought to play an important role in osmotic adjustment. Numerous genes that are involved in stress tolerance mechanisms (e.g., genes encoding osmolytes, antioxidant enzymes, components of calcium signaling, and other regulatory signaling factors) are also upregulated in response to salt stress. By and large, tolerance of plant to salinity stress is a synchronized action of multiple stress responsive genes, which also crosstalk with other components of stress signal transduction pathways.

4.1
Introduction

Being sessile organisms, plants cannot escape but often face and are compelled to grow under various unfavorable conditions in natural environment, such as drought, salinity, chilling, freezing, high and low temperature, flooding, or extreme light, and so on, which are collectively known as abiotic stresses. Any of these extreme conditions can delay growth and development, reduce productivity, and, in extreme

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cases, lead to plant death [1]. Abiotic stress conditions cause extensive losses to agricultural production worldwide. Soil salinity is among one of the major abiotic stresses that adversely affects crop productivity and quality. Salinity affects nearly 20% of the world's cultivated area and about half the world's total irrigated lands [2]. By and large, reduction in growth and yield caused by salinity is mainly due to salt-induced water stress, nutritional imbalance, specific ion toxicity toward photosynthesis, hormonal imbalance, and generation of reactive oxygen species (ROS), which may cause membrane destabilization [3–10]. Soil salinity is characterized by the toxic levels of chlorides and sulfates of sodium in the soil. It is mainly caused by irrigation with groundwater of high salt content, improper drainage, entry of seawater during cyclones in coastal areas, and salt accumulation in arid and semiarid regions due to high evaporative demand and insufficient leaching of ions owing to inadequate rainfall [11, 12]. Excess salts in the soil reduce permeability of soils [13]. Salt-affected soils usually contain a variety of inorganic salts with cations such as Na^+, Ca^{2+}, Mg^{2+}, and K^+ and anions such as Cl^-, SO_4^{2-}, HCO_3^-, CO_3^{2-}, and $NO3^-$ [14] that adversely affect plant growth and productivity causing ion toxicity or osmotic effect on plants [5, 15].

The most important effects of salinity on plants are lowering of water potential, specific ion toxicity (sodium and chloride), and interference with the uptake of essential nutrients. The interference with the uptake of essential nutrients, however, may not be considered important because it has no immediate effect due to mobile reserve nutrients present in plants [16]. Decreased water potential and ion toxicity are considered important factors in reducing plant growth under salt stress. Salt-sensitive cultivars accumulate ions more quickly than salt-tolerant cultivars leading to leaf death and progressive death of plant [9]. Ion imbalances due to ion accumulation caused by salt stress show their negative effects by reducing shoot and root growths and increasing some amino acids including proline [17].

Sodium is an essential micronutrient for some of the plants, but most crop plants are natrophobic. Salinity causes nutritional constraints as it decreases uptake of phosphorus, potassium, nitrate, and calcium, while leading to ion cytotoxicity and osmotic stress [2]. Salinity may also cause denaturation of functional and structural proteins [18]. Under salinity, ions like Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the function of these proteins. Ionic toxicity, osmotic stress, and nutritional defects under salinity lead to metabolic imbalances and oxidative stress. Plant salt tolerance mechanisms can be grouped into cellular homeostasis (including ion homeostasis and osmotic adjustment), stress damage control (repair and detoxification), and growth regulation [2]. Antioxidant enzyme activities are usually affected by salinity and used as indicators of oxidative stress in plants [19]. To protect against oxidative stress, plant cells produce both antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT) and nonenzymatic antioxidants such as ascorbate, glutathione, and tocopherol [19, 20]. The presence of high concentration of salt in the growth medium also often results in accumulation of low molecular mass compounds such as proline and glycine betaine (GB), commonly termed compatible solutes, which do not interfere with the normal biochemical reactions [4, 13, 21].

4.2
Effects on Plant Growth and Development

Salinity affects almost every aspect of the physiology and biochemistry of plants and significantly reduces growth and yield. High exogenous salt concentrations affect seed germination, lead to water deficit, and cause ion imbalance of the cellular ions resulting in ion toxicity and osmotic stress [22]. The most important process that is affected in plants, growing under saline conditions, is photosynthesis. Most of the cultivated plants are sensitive to salt stress, in which NaCl salinity causes reduction in carbohydrates that are needed for cell growth. Carbohydrates are supplied mainly through the process of photosynthesis, and photosynthesis rates are usually lower in plants exposed to salinity and especially to NaCI [5, 23]. This would further lead to restriction in water availability and imbalance in nutrient uptake by plants [24, 25] with inhibition in seed germination due to ionic disturbance and osmotic and toxic effects [26, 27]. Burman *et al.* [28] emphasize that the reduction of photosynthesis due to salinity is primarily by a decrease in stomatal conductance (g_s), while Wilson *et al.* [29] and Yang and Lu [30] suggested it is due to a decrease in CO_2 diffusion to the chloroplast. Reduced photosynthesis under salinity is attributed not only to stomata closure leading to a reduction in intercellular CO_2 concentration but also to non-stomatal factors such as depression in PSII activity, electron transport, photophosphorylation activity [31], photosynthetic enzymes, chlorophyll, and carotenoids. However, it is a well-accepted fact that photosynthetic activity decreases when plants are grown under saline conditions leading to reduced growth and productivity [32]. Stomatal conductance (g_s) can be reduced with increasing environmental salinity. Like drought stress, salt stress involves a decrease in soil water potential. Stomata are sensitive to changes in soil water potential and usually close during the times of drought and salt stress [33]. A decrease in g_s reduces incoming CO_2 and thus can reduce photosynthesis rates. Salinity reduces the ability of plants to utilize water and causes not only a reduction in growth rate but also changes in plant metabolic processes [9].

High salinity causes both hyperionic and hyperosmotic stress effects in plants and the consequence can be fatal [7]. Osmotic effects are caused by salt-induced decrease in the soil water potential. Salinity results in a reduction in K^+ and Ca^{2+} content and an increased level of Na^+, Cl^-, and SO_4^{2+}, which forms its ionic effects [34]. Reduction in biomass, reduction in photosynthetic capacity, and changes in leaf water potential and leaf turgor have been reported to have a cumulative effect attributed to salinity stress [35]; it is also clear that several soil and other environmental factors do influence plant growth under salinity conditions [36]. Salt ion toxicity has numerous deleterious effects on plants such as denaturing cytosolic enzymes and facilitating the formation of reactive oxygen species that can damage membranes, proteins, and nucleic acids [2, 19]. Lipid peroxidation, induced by free radicals, is also important in membrane deterioration [22, 37–39]. Soil salinity also increases P, Mn, and Zn and decreases K and Fe concentrations in plants [40]. Shoots are generally more sensitive to cation disturbances than roots, and there are great differences among plant species in their ability to prevent or tolerate excess salt

concentrations [41]. Increasing levels of NaCl induces a progressive absorption of Na and Cl in both shoot and root [40]. Excessive Na concentration in the plant tissue hinders nutrient balance and osmotic regulation, and causes specific ion toxicity [24, 25]. Accumulation of Cl in the root tissue is disruptive to membrane uptake mechanisms, and these result in increased translocation of Cl to the shoots [42]. Cordovilla *et al*. [43] reported that NaCl decreased N concentration in the shoot tissues. Salinity has a negative interference in the nitrogen acquisition and utilization [44], reason being the antagonism between Cl^{-1} and $NO_3{}^{-1}$ [45]. The mechanism of plant adaptation required to survive in saline conditions is the same in all the plants. However, adaptations are at their extreme in halophytes, but can also be found in different degrees in glycophytes [16]. Variation in salt tolerance of glycophytes occurs between and within species and has been quantified for many crops [46].

4.3 Ionic Stress

Restriction of Na^+ entry into the root cells and then into the transpirational stream is crucial to prevent a buildup of toxic levels of salt in the shoot. Plants (both glycophytes and halophytes) must exclude about 97% of the Na^+ present in the soil at the root surface itself to prevent toxic levels of Na^+ accumulation in the shoots [11]. Sodium entry into the transpirational stream depends upon the amount of Na^+ uptake by Na^+ and nonspecific cation transporters and the proportion of water entry in the apoplastic/bypass pathway into the xylem. Na^+ in the soil gains initial entry into the cells of the root epidermis and cortex. The casparian strip in the endodermis plays a crucial role in preventing apoplastic Na^+ influx into the root stele [12]. Increased Na^+ accumulation in the plant due to high concentrations of Na^+ in the soil causes ionic stress in plants [47]. The complexity of the plant response to salt stress can be partially explained by the fact that salinity imposes salt toxicity in addition to osmotic stress [4]. Accumulation of sodium in the cytoplasm is prevented by restricting its uptake across the plasma membrane and by promoting its extrusion or sequestration in halophytes [4]. High salt concentrations (>400 mM) inhibit the activities of most enzymes because of perturbation of the hydrophobic–electrostatic balance between the forces maintaining protein structure [47]. However, toxic effects on cells occur at much lower salt concentrations (about 100 mM) [48]. Many studies have demonstrated that toxic effects of Na^+ in the cytosol is not the only reason of Na^+ toxicity but is also caused by disruption in K^+ homeostasis, possibly owing to the ability of Na^+ competing for K^+ binding sites [47]. Substantial advancement has been made in understanding ion homeostasis [4, 49, 50] and ion transporters are considered to play a significant role in salt tolerance. Bartels and Sunkar [47] suggested three mechanisms that prevent excess Na^+ accumulation in the symplast of plant cells: (1) restriction of Na^+ infiltration and entry into plants by Na^+ transporters, whose molecular identity is unknown; (2) compartmentalization of Na^+ in the vacuole; and (3) extrusion of Na^+: cytosolic Na^+ can be transported

back to the external medium or the apoplast via plasma membrane Na^+/H^+ antiporter activity.

4.3.1 Ion Selectivity

A major feature of the solute transport by plants in saline conditions is the degree of selectivity, particularly between potassium and sodium [51]. One of the most important physiological mechanisms of salt tolerance is the selective absorption of K^+ by plants from the saline media [52]. Halophytic or salt-tolerant species differ from salt-sensitive ones in having restricted uptake or transport of Na^+ and Cl^- to the leaves despite an effective compartmentalization of these ions. This is critical in preventing the buildup of toxic ions in cytoplasm [6, 9]. Ion imbalance, particularly that caused by Ca^{2+} and K^+, is the most important and widely studied phenomenon affected by salt stress, which is directly influenced by the uptake of Na^+ and Cl^- ions [9, 53]. Maintaining better concentrations of K^+ and Ca^{2+} and limiting the Na^+ uptake are vital for the salt stress tolerance in plants [54]. Higher K^+/Na^+ or Ca^{2+}/Na^+ ratios are characteristic of the tissue salt tolerance and are often used as a screening criterion for salt tolerance [6, 55, 56].

4.3.2 Na^+ Exclusion

Control of Na^+ levels at the entry point is crucial to avoid ionic stress [9]. The Na^+ uptake across the plasma membrane has been attributed to low Na^+ permeability properties of systems that transport essential K^+ [4]. The mechanism involves transport of Na^+ to the leaves and subsequently excreted out of the plant body, thereby reducing Na^+ concentration in plant tissues. Na^+ that is translocated in the transpiration stream is deposited and its concentration increases with time. This leads to much higher Na^+ concentrations in older leaves than those in younger leaves [57]. Na^+ exclusion is the most important adaptive strategy regulating the internal Na^+ load of halophytes. For instance, about 98% of Na^+ was reported to be excluded in the mangrove species *Avicennia marina* growing in 500 mM NaCl [58]. In perennial plants, exclusion is particularly important and it is more vital to regulate the incoming Na^+ load in the plant body [4, 59]

4.3.3 Na^+ Sequestration

Besides, efficiently excluding Na^+, halophytes effectively compartmentalize Na^+ in their vacuole that is why they can survive in high concentration saline soil. Conversely, glycophytes exclude Na^+ but cannot compartmentalize it [57]. In plants, the central vacuole plays a vital role in the regulation of cytoplasmic ion homeostasis. Exclusion of excess Na^+ from the cytoplasm and its accumulation in the vacuole represents one of the adaptive mechanisms during salt stress [47]. The vacuolar sodium sequestration or

compartmentalization of Na^+ into the vacuole is mediated by vacuolar Na^+/H^+ antiports at the tonoplast and uses the proton motive force generated by the vacuolar H^+ translocating enzymes, H^+-adenosine triphosphatase (ATPase) and H^+-inorganic pyrophosphatase (PPiase), to couple the downhill movement of H^+ with the uphill movement of Na^+ against the electrochemical potential [60].

4.4 Osmotic Stress

High salinity disturbs uptake and conductance of water. Most commonly, the stress is caused by high Na^+ and Cl^- concentrations in the soil solution [4]. An increase in the external salinity decreases water flow into the plant, limits water uptake to cells, and reduces turgor potential and cell volume [61]. Altered water status most likely brings about initial growth reduction; this reduction does not appear to depend on salt concentration in the growing tissues, rather it is a response to osmolarity of the external solution [9]. Plants maintain a high cytosolic K^+/Na^+ ratio under optimal conditions. Salt stress-induced decrease in the K^+/Na^+ ratio is inimical to cellular biochemical processes. In addition, K^+ provides necessary osmotic potential for water uptake by plant cells [62]. Thus, K^+ uptake is pivotal to cell turgor and maintenance of biochemical processes under salinity. In plants, Na^+ competes with K^+ for uptake under saline conditions. Membrane disorganization, reactive oxygen species, metabolic toxicity, inhibition of photosynthesis, and attenuated nutrient acquisition are factors that initiate more catastrophic events [63]. Salt stress that affects water supply leads to changes in stomatal opening that can, if stress persists, set in motion a chain of events originating from a decline in the leaf internal CO_2 concentration, consecutively inhibiting the carbon reduction cycle, light reactions, energy charge, and proton pumping [64]. Other pathways are affected by increased shuttling of carbon through the photorespiratory cycle [65, 66]. Eventually, carbon and nitrogen allocation and storage require readjustment, reactions that lead to the consumption of reducing power become favored, and development and growth may get altered [67].

4.4.1 Osmotic Adjustment

Under osmotic stress, plants need to maintain internal water potential below that of soil and maintain turgor and water uptake for growth [7]. This requires an increase in osmotica, either by uptake of soil solutes or by synthesis of metabolic (compatible) solutes [68]. To accommodate the ionic balance in vacuoles, cytoplasm accumulates osmotically active compounds called osmolytes in order to lower the osmotic potential. These compounds are referred to as compatible metabolites because they do not apparently interfere with the normal cellular metabolism [21]; rather, they replace water in biochemical reactions [68]. These organic solutes protect plants from salt stress by (i) osmotic adjustment, which helps in turgor maintenance; (ii) detoxification of reactive oxygen species; and (iii) stabilization of the quaternary

structure of proteins [66]. Although some compatible solutes are essential elemental ions, such as K^+, a majority are organic solutes [69], and accumulation of osmolytes varies between plant species. Generally, these molecules are not highly charged, but are polar, highly soluble, and have a larger hydration shell. Such molecules will be preferentially solubilized in the bulk water of the cell where they could interact directly with the macromolecules [70]. Compatible solutes consist of simple sugars (mainly fructose and sucrose), sugar alcohols (such as mannitol, sorbitol, and inositol) and complex sugars (such as trehalose and fructans), and quaternary amino acid derivatives or charged metabolites such as glycine betaine and proline. There may be more than one function for a particular solute [71, 72], and according to the results from *in vitro* experiments [73], different compatible solutes seem to have different functions [67]. The main function of compatible solutes may be stabilization of proteins, protein complexes, or membranes under environmental stress. In *in vitro* experiments, compatible solutes at high concentrations have been found to reduce the inhibitory effects of ions on enzyme activity [74], to increase thermal stability of enzymes [75], and to prevent dissociation of the oxygen evolving complex of photosystem II (PSII) [76].

Genes involved in osmoprotectant biosynthesis are upregulated under salt and drought stresses [77, 78]. Enhanced tolerance to salt stress was observed in transgenic plants engineered to overaccumulate mannitol [71, 79], glycine betaine [80–82], and proline [83, 84]. Transgenic rice plants expressing a peroxisomal betaine aldehyde dehydrogenase of barley accumulated fewer Na^+ and Cl^- ions and more K^+ ions [81].

4.4.1.1 **Soluble Sugars**

Numerous studies have shown that under salt stress conditions plant accumulate nonstructural carbohydrates (sucrose, hexoses, and sugar alcohols), though in varying degrees in different plant species [47]. A strong correlation between sugar accumulation and osmotic stress tolerance has been reported [85]. Of the various organic osmotica, sugars contribute up to 50% of the total osmotic potential in glycophytes subject to saline conditions [86]. The accumulation of soluble carbohydrates in plants has been widely reported as a response to salinity or drought, despite a significant decrease in net CO_2 assimilation rate [87]. Carbohydrates such as sugars (glucose, fructose, sucrose, and fructans) and starch accumulate under salt stress [88], playing a leading role in osmoprotection, osmotic adjustment, carbon storage, and radical scavenging. A decrease in starch content and an increase in both reducing and nonreducing sugars and polyphenol levels have been reported in leaves of *Bruguiera parviflora* [88]. In leaves of tomato, the contents of soluble sugars and total saccharides are increased significantly, but the starch content is not affected [89]. Ashraf and Tufail [90] found that although total soluble sugar content increased significantly in all five sunflower lines with increasing salt concentrations, the salt-tolerant lines had generally greater soluble sugars than the salt-sensitive ones. On the contrary, in safflower the pattern of accumulation of soluble sugars differed, even within salt-tolerant accessions, while one salt-tolerant line accumulated a high content of soluble sugars, another line was salt tolerant despite solute accumulation similar to that in

salt-sensitive accessions [91]. Ashraf [92] compared salt-tolerant wild populations with cultivated populations of *Melilotus indica* and *Eruca sativa* and found that *M. indica* had significantly higher soluble sugars in their leaves than the latter salt-sensitive populations at varying levels of salt concentrations. It has been reported that trehalose, a disaccharide that accumulates in many organisms, acts as both osmolyte and osmoprotectant under various abiotic stresses [93], protects membranes and proteins in cells exposed to stresses such as salinity and drought that cause water deficit [94], and reduces aggregation of denatured proteins [95]. Recently, Yamada *et al.* [96] have reported that trehalose has a suppressive effect on apoptotic cell death. It has also been reported that many higher plants possess trehalose activity, which is perhaps responsible for rapid degradation of any trehalose synthesized [47]. *Arabidopsis thaliana* has at least one gene that encodes trehalose-6-phosphate phosphatase required for trehalose synthesis, but the physiological role of this enzyme is not yet clear [97].

4.4.1.2 **Polyols**

Polyols, the polyhydric alcohols, which exist in both acyclic and cyclic forms, are involved in osmoregulation and are believed to play a role in plant salt tolerance [98]. The most common polyols found in plants consist of acyclic forms, mannitol, glycerol, sorbitol, and cyclic (cyclitols) forms ononitol and pinitol. Generally, they are thought to be accumulated in the cytoplasm of some halophytes to overcome the osmotic disturbances caused by high concentrations of inorganic ions compartmentalized in vacuoles [99]. Besides their role in osmoregulation, polyols also function as oxygen radical scavengers. For example, mannitol was found *in vitro* to act as a scavenger of reactive oxygen species [100], thereby protecting proteins from oxidative damage in drought-stressed plants [101]. Smirnoff [18] found that mannitol, sorbitol, glycerol, ononitol, and pinitol were all active scavengers. Polyols make up a considerable percentage of all assimilated CO_2 as scavengers of stress-induced oxygen radicals [102].

Transgenic tobacco plants synthesize mannitol-1-phosphate from fructose-6-phosphate. Although *Escherichia coli mtlD* gene-transformed tobacco plants and wild type have similar growth in the absence of salt stress, transgenic plants have growth advantage over the wild type in the presence of 250 mol m^{-3} of salt [103]. Binzel *et al.* [104] found that tobacco cells adapted to 428 mM of NaCl could maintain cytosolic Na^+ and Cl^- level less than 100 mM. Though mannitol only partially decreases the amount of inorganic ion accumulation in the cytosol, its protective effect as a compatible solute may be sufficient to give marginal growth advantage to transformed plants [70]. Su *et al.* [105] obtained three rice transgenic lines with bacterial *mtlD* and demonstrated that biosynthesis and accumulation of mannitol in plants are correlated with salt stress tolerance of plants. These solutes are widely believed to function as a protector or stabilizer of enzymes or membrane structures that are sensitive to dehydration- or ion-induced damage [70].

Pinitol and other *O*-methyl inositols may play an important role both in intracellular osmotic adjustment between the vacuole and the cytoplasm and in scavenging free radicals [23]. Pinitol and ononitol are found stored in a variety of species, which are consistently exposed to saline conditions or accumulate in tolerant species when exposed to salt stress [17]. Pinitol increases considerably in plants in response to water

deficit [106]. It has been reported that pinitol was accumulated in *Honkenya peploides* [107] and *Sesbania aculeate* [108] exposed to salt stress; however, the inositol content in the *S. aculeate* leaves remained unchanged under salt stress [108]. A significant accumulation of pinitol and ononitol in alfalfa was found by Fougere *et al.* [109], suggesting that pinitol might contribute to tolerance to salt stress. Facultative halophyte such as *Mesembryanthemum crystallinum* accumulates these compounds only when subjected to water and salinity stresses. The proposed synthetic pathway consists of methylation of myo-inositol to the intermediate ononitol followed by epimerization to pinitol [110]. An inositol methyl transferase (*Imt*) cDNA was isolated from transcripts induced in *Mesembryanthemum* plants by NaCl [111]. Transgenic tobacco for inositol methyl transferase has been obtained [112]. Similar to plants transformed with mannitol-1-phosphate dehydrogenase, growth of wild-type and *Imt*-transformed plants is not distinguishable in the absence of stress, but the transgenic plants have growth advantage over wild type in the presence of salt stress [70].

4.4.1.3 **Proline**

Many plants (not all) synthesize and accumulate proline, a major osmoprotectant osmolyte, in response to various stresses including salinity stress [113]. Generally, proline accumulation occurs in cytosol where it contributes significantly to the cytoplasmic osmotic adjustment [114]. In higher plants, the amino acid proline is synthesized by glutamic acid by the actions of two enzymes, pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). Overexpression of *P5CS* gene in transgenic tobacco resulted in increased production of proline and salinity/drought tolerance [115]. The exogenous application of proline also provided the osmoprotection and facilitated the growth of salinity-stressed plants. Proline can also protect cell membranes from salinity-induced oxidative stress by upregulating activities of various antioxidants [116]. It is reported that the salt stress enhances proline utilization in the apical region of barley roots [117]. The function of proline is thought to be an osmotic regulator under water stress, and its transportation into cells is mediated by a proline transporter. However, recently, Ueda *et al.* [118] have reported that altered expression of barley proline transporter (*HvProT*) causes different growth responses in *Arabidopsis*, as it leads to a reduction in biomass production and decreased proline accumulation in leaves. Impaired growth of *HvProT*-transformed plants was restored by exogenously adding proline, which implies that growth reduction was caused by a deficiency of endogenous proline. Petrusa and Winicov [119] and Fougere *et al.* [109] reported that salt-tolerant alfalfa plants rapidly increased proline content twofold in the roots, whereas the increase was rather slow in salt-sensitive plants. Ahmad *et al.* [120] observed that salt-tolerant ecotypes of *Agrostis stolonifera* accumulated more proline in response to salinity than the salt-sensitive ecotypes. Moderately salt-tolerant plants of *Brassica juncea* showed a higher degree of osmotic adjustment in the leaves and a higher critical point concentration of NaCl, at which the endogenous level of free proline rose sharply, than the moderately salt-sensitive genotypes [121]. Higher proline accumulation was found in salt-tolerant *B. juncea* plants with better growth than the control [122].

Transgenic approaches provided direct evidence for the role of proline during osmotic stress. Different strategies were employed to manipulate proline biosynthesis including overexpression of P5CS in tobacco, rice, and *Arabidopsis* plants, overexpression of OAT, expression of a feedback inhibition-insensitive form of *P5CS*, and antisense suppression of proline oxidation by ProDH [83, 123]. All approaches resulted in elevated proline pools and improved osmotic stress tolerance. However, contrary to these observations, a study on antisense ProDH transgenic *Arabidopsis* plants observed that these plants accumulated proline but showed no change in osmotic stress tolerance [124].

4.4.1.4 Glycine Betaine

Glycine betaine is thought to protect the plant by maintaining the water balance between the plant cell and the environment and by stabilizing macromolecules [1]. This organic compound is mainly localized in chloroplasts and plays a vital role in chloroplast adjustment and protection of thylakoid membranes, thereby maintaining photosynthetic efficiency and plasma membrane integrity [69]. Plants synthesize glycine betaine via a two-step oxidation of choline: choline → betaine aldehyde → glycine betaine [125]. The first reaction is catalyzed by a ferredoxin-dependent choline monooxygenase (CMO) and the second step by an NAD^+-dependent betaine aldehyde dehydrogenase (BADH) [1]. Although other pathways such as direct N-methylation of glycine is also known, the pathway from choline to glycine betaine has been identified in all GB accumulating plant species [126]. Glycine betaine accumulation is associated with upregulated CMO and BADH gene expression concomitantly leading to elevated enzymatic activity [47].

GB accumulates in response to stress in many crops, including spinach, barley, tomato, potato, rice, carrot, and sorghum [127]. Murata *et al.* [128] reported that GB protects the photosystem II complex by stabilizing the association of the extrinsic PSII complex proteins under salt stress. The concentration of GB in plant species that utilize it as a compatible osmoticum is inconsistent; for example, in sorghum it is as much as 10-fold compared to maize. GB-deficient genotypes of both species have also been identified [68]. Accumulation of glycine betaine under saline conditions is also reported to be high in some salt-tolerant plants of mulberry in comparison to sensitive ones [129]. Saneoka *et al.* [130] reported that glycine betaine-containing lines of maize exhibited less shoot growth inhibition under saline conditions than deficient lines. Glycine betaine accumulation marginally improves osmotic stress tolerance in transgenic plants [131]. The levels of glycine betaine thus far obtained by engineering are low, and the increments in stress tolerance are small [132]. The major factors that limit the accumulation of glycine betaine are the available choline as the substrate for the reaction and its transport from the chloroplast (where it is synthesized) to the cytosol [1, 133, 134]. Foliar application of GB resulted in a significant improvement in salt tolerance of rice plants [135] and tomato plants subjected to either salt stress or high temperatures resulted in about 40% increase in fruit yield compared to untreated plants [136]. However, there are also few studies suggesting a lack of such positive effects or even apparent negative effects of exogenous GB on plants growing under stress conditions [68].

4.5
Salt Stress-Induced Proteins

Osmotic stress induces several proteins in vegetative tissues of higher plants, which are related to late-embryogenesis abundant (LEA) proteins. The correlation between LEA protein accumulation in vegetative tissues and stress tolerance in various plant species indicates its protective role under dehydration stress [137]. Proteins that accumulate in plants grown under saline conditions may provide a storage form of nitrogen that is reutilized when stress is over [138] and may play a role in osmotic adjustment. Proteins may be synthesized *de novo* in response to salt stress or may be present constitutively at low concentration and increase when plants are exposed to salt stress [139]. Hasegawa *et al*. [4] reported that a number of proteins induced by salinity are cytoplasmic that can cause alterations in cytoplasmic viscosity of the cells. A higher content of soluble proteins has been observed in salt-tolerant cultivars of barley, sunflower, finger millet, and rice [23]. Soluble protein increases at low salinity and decreases at high salinity in mulberry cultivars [129]. Despite the reports on decrease in soluble-protein content in response to salinity, Ashraf and Fatima [91] found that salt-tolerant and salt-sensitive accessions of safflower did not differ significantly in leaf soluble proteins. Singh *et al*. [138] detected a 26 kDa protein called osmotin during characterization of salt-induced proteins in tobacco. An osmotin-like protein was found increased in salt-stressed *M. crystallinum* compared to nonstressed plants [140]. In barley, two 26 kDa polypeptides, not immunologically related to osmotin, identified as germin were increased in response to salt stress [141]. Lopez *et al*. [142] found a 22 kDa protein in response to salt stress in radish and in finger millet (*Eleusine coracana*), and Uma *et al*. [143] found 54 kDa and 23–24 kDa proteins responsible for salt or drought tolerance.

Engineered rice plants overexpressing a barley *LEA* gene, *HVA1*, under the control of the rice actin 1 promoter, showed better stress tolerance under 200 mM NaCl than the wild type [144]. *Arabidopsis* LEA-like stress proteins are encoded by *COR* genes (*RD29A*, *COR47*, *COR15*, *KIN1*, and *KIN2*) that are induced by cold, dehydration (due to water deficit or high salt), or ABA. Promoter analysis of the *COR* genes showed that many of them contain dehydration-responsive elements (DRE) or C-repeat (CRT) and ABA-responsive elements or ABREs. Transcription factors that regulate the LEA-like genes include CBFs (C-repeat binding proteins, also known as dehydration-responsive element binding proteins, DREBs) and bZIP proteins [12]. The expression of *COR* genes is regulated by both ABA-dependent and -independent pathways [145]. Constitutive overexpression of *CBF3* or stress-induced expression of *CBF3* driven by the *RD29A* promoter resulted in enhanced expression of *COR* genes under salt stress in transgenic *Arabidopsis* and also conferred higher osmotic stress tolerance [146]. *CBF3* overexpression in *Arabidopsis* also resulted in elevated accumulation of proline and total soluble sugars, including sucrose, raffinose, glucose, and fructose. The increase in proline levels is considered due to the increased expression of the key proline biosynthetic enzyme Δ1-pyrroline-5-carboxylate synthase [147]. Hence, LEA-like proteins appear to protect plants under salt stress. Osmotic or salt stress-induced calcium signals may activate the LEA-like genes through DREB2

transcription factors, while salt stress-induced ABA accumulation appears to induce the genes through ABA-responsive element binding factors [77, 148].

4.6 Oxidative Stress

4.6.1 Reactive Oxygen Species

A secondary effect of salt stress is the increase in the production of ROS, which consist of singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) [18, 149]. The oxidative stress arising under environmental stresses including salinity and may exceed the scavenging capacity of the natural defense system of the plant. ROS are predominantly generated in the chloroplast either by direct transfer of excitation energy from chlorophyll to produce singlet oxygen or by univalent oxygen reduction at photosystem I, in the Mehler reaction [150], and to some extent in mitochondria. Chloroplasts are the first targets in plant cells since this is the major site of ROS production. The increased concentration of ROS inhibits the ability to repair damage to photosystem II and inhibits the synthesis of the D1 protein. Stress-enhanced photorespiration and NADPH activity also contributes to an increased H_2O_2 accumulation, which may inactivate enzymes by oxidizing their thiol groups. The toxicity of H_2O_2 is not due to its reactivity as such, but requires the presence of a metal reductant to form the highly reactive hydroxyl radical, which potentially reacts with all biological molecules. Transition metals such as cuprous and ferrous ions may be released from enzymes and electron carriers during stress and promote the Fenton reaction to produce highly reactive hydroxyl radicals, which extensively oxidize proteins, lipids, and nucleic acids [151]. Hence, ROS detoxification forms a crucial defense against salt stress.

4.6.2 Oxidative Stress Management

4.6.2.1 Antioxidant Enzymes

Plants have developed strategies to keep the concentrations of ROS under tight control through detoxification/scavenging by antioxidant enzymes. ROS detoxification mechanisms can be broadly divided into nonenzymatic and enzymatic mechanisms. Major nonenzymatic antioxidants include ascorbate (vitamin C) and glutathione (GSH) in plants, although tocopherol (vitamin E), flavonoids, alkaloids, and carotenoids can also act as antioxidants. Enzymatic mechanisms include SOD, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), POX, CAT, guaiacol peroxidase (GOPX), glutathione reductase (GR), and glutathione-*S*- transferase (GST) [19, 149]. The alleviation of oxidative damage and increased resistance to environmental stresses is often correlated with an efficient antioxidative system [18, 152, 153]. Increase in the activity

of SOD, APX, GR, DHAR, CAT, and POX in response to salinity stress, as well as higher antioxidant activity in tolerant species/varieties, has also been reported by various workers [4]. Sairam and Srivastava [154] reported comparatively higher Cu/Zn-SOD, Fe-SOD, APX, and GR activity in chloroplastic fraction and Mn-SOD in mitochondrial fraction in tolerant wheat genotypes in response to salt stress. Hernandez *et al.* [37] reported NaCl-induced enhanced mRNA expression and activity of Mn-SOD, APX, GR, and MDHAR in tolerant pea cv. Granada, while in salinity-sensitive cv. Chillis, no significant changes in activity and mRNA levels of the above enzymes were observed. Significantly higher constitutive concentrations of catalase and α-tocopherol were found in salt-tolerant cotton plants than in salt sensitive lines [155]. Salt stress caused a considerable increase in the activities of peroxidase and glutathione reductase in the salt-tolerant cultivars, whereas the activities of these enzymes remained unchanged or decreased in the salt-sensitive cultivars. The salt-tolerant cultivars also had a lower oxidized/reduced ascorbate ratio and a higher reduced/oxidized glutathione ratio than the salt-sensitive lines under saline conditions. Lipid peroxidation in the salt-sensitive lines increased more than in the salt-tolerant lines under salt stress, suggesting that high levels of antioxidants and an active ascorbate-glutathione cycle are associated with salt tolerance in cotton. Shalata and Tal [156] assessed the possible involvement of the antioxidant system in the salt tolerance of cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* and reported that in the wild type the constitutive level of lipid peroxidation and activities of catalase and glutathione reductase were lower, whereas the activities of SOD, ascorbate peroxidase, and dehydroascorbate reductase were inherently higher than those in the cultivated tomato species. Mittova *et al.* [157] correlated the better protection of wild salt-tolerant tomato (*L. pennellii*) root plastids from salt-induced oxidative stress with increased activities of SOD, APX, and POD (guaiacol peroxidase).

Overproduction of SOD, APX, and catalase has been shown to improve oxidative stress tolerance in transgenic plants [158, 159]. Roxas *et al.* [160] reported overexpression of a tobacco glutathione-*S*-transferase (GST) and glutathione peroxidase (GPX) in transgenic tobacco seedlings under a variety of stresses. Salt stress treatment inhibited the growth of the wild type and caused increased lipid peroxidation, while GST-transformed seedlings did not lead to increased lipid peroxidation. GST/GPX overexpression provides increased glutathione-dependent peroxidase scavenging and alterations in glutathione and ascorbate metabolism, leading to reduced oxidative damage. Studies on transgenic rice overexpressing yeast Mn-SOD showed increased levels of ascorbate peroxidase and chloroplastic SOD in the transformed rice compared to the wild type. The transformed rice also showed more salinity tolerance than the wild type [161].

4.7
Calcium Signaling and SOS Pathways

Extreme salinity results in increased cytosolic Ca^{2+} that is transported from the apoplast and the intracellular compartments. Cytosolic Ca^{2+} oscillations occur

within 5–10 s of salt stress, persist up to 1–10 min, and, hence, are thought to be one of the earliest events in salt signaling [162]. Thus, it is essential to analyze how such Ca^{2+} signatures are generated by a salt stress signal and what are the components downstream that decode salt stress-specific Ca^{2+} signatures [12]. Cytosolic Ca^{2+} signatures can be the net result of influx and efflux of Ca^{2+}. Calcium efflux occurs through Ca^{2+} ATPases and H^+/Ca^{2+} antiporters, while influx is controlled by Ca^{2+}-permeable ion channels [163]. The transient increase in cytosolic Ca^{2+} due to salt stress initiates the stress signal transduction leading to salt adaptation. This Ca^{2+} release is primarily of extracellular source (apoplastic space) and also takes place from the activation of phospholipase C (PLC), leading to hydrolysis of phosphotidylinositol-4,5 bisphosphate (PIP_2) to inositol (1,4,5)-triphosphate (IP_3) and subsequent release of Ca^{2+} from intracellular Ca^{2+} stores [164, 165]. The Ca^{2+} binding proteins sense and relay the information downstream to initiate a phosphorylation cascade leading to the regulation of gene expression [166].

In *Arabidopsis*, osmotic stress (NaCl or sorbitol) induces the synthesis of IP_3 to significantly higher levels within 1 min of stress initiation, and it continues to increase for more than 30 min. Treatment with U-73122, an inhibitor of PLC, blocked IP_3 accumulation. The temporal pattern of IP_3 accumulation is similar to that observed for stress-induced calcium mobilization, implicating IP_3 in salt stress-induced Ca^{2+} signaling [167, 168]. In cell cultures of *Arabidopsis*, a few seconds of osmotic stress (caused by dehydration, mannitol, or NaCl) led to a rapid and transient increase in IP_3 and expression of dehydration-inducible genes (*RD29A*/*LTI78*/*COR78* and *RD17*/*COR47*). This response was abolished when the cells were treated with inhibitors of PLC, such as neomycin and U73122, indicating the involvement of PLC and IP_3 in hyperosmotic stress signaling [168]. Osmotic stress caused by NaCl/mannitol/sorbitol significantly increases cellular PIP_2 synthesis [167, 169]. Consistent with this, it has been shown that a *PLC* gene is also upregulated by osmotic stress [170]. Salt stress-induced PIP_2 synthesis and cleavage into IP_3 may help in delayed Ca^{2+} signaling. Genetic evidence for the implication of IP_3 signaling in abiotic stresses including salinity stress came from the analysis of the *FRY1* locus of *Arabidopsis*. *FRY1* encodes an inositol polyphosphate 1-phosphatase, which functions in the catabolism of IP_3. Upon ABA treatment, *fry1* mutant plants accumulated more IP_3 than the wild-type plants. In wild type, IP_3 accumulation was transiently induced by ABA, while in *fry1* IP_3 accumulation was persistent, which implies that IP_3 catabolism is mediated by FRY1. The *fry1* mutant is hypersensitive to ABA and salinity stress [171]. The *Arabidopsis SAL1* gene, a homologue of *FRY1* conferred increased salt tolerance to yeast cells [172]. These results showed that IP_3 transient induced by salt and ABA is necessary for stress tolerance. Besides IP_3-gated Ca^{2+} channels, stretch-/mechanosensitive Ca^{2+} channels may also be involved in primary Ca^{2+} oscillations, as these Ca^{2+} channels can be activated immediately by a change in cell volume/turgor in salt-stressed cells. Hence, salt stress-induced IP_3 oscillations are an integral part of Ca^{2+} signaling in salt stress.

Wu *et al.* [173] commenced a mutant screen for *Arabidopsis* plants, which were oversensitive to salt stress. As a result of this screen, three genes *SOS1*, *SOS2*, and

SOS3 (Salt Overly Sensitive) were identified. *SOS3* gene (also known as *AtCBL4*) encodes a calcineurin B-like protein (CBL, Ca^{2+} sensor), which is a Ca^{2+} binding protein and senses the change in cytosolic Ca^{2+} concentration and transduces the signal downstream. The SOS pathway results in the exclusion of excess Na^+ ions out of the cell via the plasma membrane Na^+/H^+ antiporter and helps in reinstating cellular ion homeostasis. The discovery of SOS genes paved the way for elucidation of a novel pathway linking the Ca^{2+} signaling in response to a salt stress [77, 174]. SOS genes (*SOS1*, *SOS2*, and *SOS3*) were genetically confirmed to function in a common pathway of salt tolerance [83]. In the SOS pathway, the salinity stress signal is perceived by an unknown hypothetical plasma membrane sensor resulting in increased cytoplasmic Ca^{2+} perturbations, which is sensed by *SOS3* followed by transduction of the signal to the downstream components. The myristoylation motif of *SOS3* results in the recruitment of SOS3–SOS2 complex to the plasma membrane, where *SOS2* phosphorylates and activates *SOS1* [175]. The *SOS1* is a Na^+/H^+ antiporter and sos1 mutant was hypersensitive to salt and showed impaired osmotic/ionic balance. The SOS pathway also seems to have other branches, which help remove excess Na^+ ions out of the cell and thereby maintain the cellular ion homeostasis. In *Arabidopsis*, Na^+ entry into root cells during salt stress appears to be mediated by AtHKTI, a low affinity Na^+ transporter, which blocks the entry of Na^+ [77, 164]. *SOS2* also interacts and activates NHX (vacuolar Na^+/H^+ exchanger) resulting in sequestration of excess Na^+ ions and pushing it into vacuoles and thereby further contributes to Na^+ ion homeostasis. Some other Ca^{2+} binding proteins such as calnexin and calmodulin (CaM) also sense the increased level of Ca^{2+} and can interact and activate the NHX. Overexpression of AtNHX1 antiporter substantially enhanced salt tolerance of *Arabidopsis* [176]. CAX1 (H^+/Ca^{2+} antiporter) has been identified as an additional target for SOS2 activity reinstating cytosolic Ca^{2+} homeostasis. This reflects that the components of SOS pathway may crosstalk and interact with other branching components to maintain cellular ion homeostasis, which helps in salinity tolerance.

So far, the main avenue in breeding crops for salt tolerance has been to reduce Na^+ uptake and transport from roots to shoots. It has been demonstrated that retention of cytosolic K^+ could also be considered as another key factor in conferring salt tolerance in plants. Recently, Zepeda-Jazo *et al.* [177] have shown that the expression of *NORC* was significantly lower in salt-tolerant genotypes. As NORC is capable of mediating, K^+ efflux coupled to Na^+ influx, suggesting that the restriction of its activity could be beneficial for plants under salt stress.

4.8 ABA-Mediated Signaling

ABA, a phytohormone that regulates plant growth and development, plays a crucial role in plants' response to abiotic stresses including salinity stress [77, 164, 178]. Salt stress induces ABA accumulation, although the amount depends upon the

tissue type. Salt stress-induced ABA accumulation is due to both ionic and osmotic stresses in roots, while that in the leaf is mainly due to osmotic stress [179]. Sequentially, turgor loss caused by osmotic stress leads to ABA synthesis and accumulation, which in turn regulates part of the cellular response to osmotic stress under salinity. ABA regulates cell water balance through stomatal regulation and genes involved in osmolyte biosynthesis, while it imparts dehydration tolerance through LEA-like genes [4, 77, 145]. The induction of osmotic stress-responsive genes imposed by salinity is transmitted through either ABA-dependent or ABA-independent pathways, though some depend only partially on ABA [180]. However, the components involved in these pathways often crosstalk through Ca^{2+} in stress signaling pathways. ABA signaling for stomatal closure and gene expression is transduced through Ca^{2+} [181]. The importance of ABA-mediated stomatal regulation in salt tolerance was revealed by the analysis of *OSM1* locus of *Arabidopsis*. Root growth of the *Arabidopsis* T-DNA insertion mutant, *osm1* (osmotic stress-sensitive mutant), was hypersensitive to NaCl or mannitol stress. Molecular cloning revealed that *OSM1* encodes a protein similar to SNARE-type mammalian syntaxins [182]. SNARE proteins are required for fusion vesicle trafficking and to control membrane Ca^{2+} and Cl^{-} channel activity and guard cell volumes [181]. Consistent with this, ABA-mediated guard cell function is impaired in the *osm1* mutant. *OSM1* is strongly expressed in roots and leaf guard cells. The *osm1* mutant showed enhanced wilting and decreased survival when salt or drought stress was imposed on soil-grown plants. Thus, OSM1 plays a critical role in root growth and in ABA regulation of stomatal responses under osmotic stresses [182].

The transcript accumulation of *RD29A* gene is reported to be regulated in both ABA-dependent and ABA-independent manner [183]. The proline accumulation in plants can be mediated by both ABA-dependent and ABA-independent signaling pathways (see Ref. [77]). The salinity stress-induced upregulation of transcript of pea DNA helicase 45 (PDH45) followed ABA-dependent pathway [184], while calcineurin B-like (CBL) protein and CBL-interacting protein kinase (CIPK) from pea followed the ABA-independent pathway [185]. The role of Ca^{2+} in ABA-dependent induction of *P5CS* gene during salinity stress has been reported [162]. Overall, the ABA-dependent pathways are involved essentially in osmotic stress gene expression. Transcriptional regulatory network of *cis*-acting elements and transcription factors involved in ABA and salinity stress-responsive gene expression has been described [165]. The ABA-dependent salinity stress signaling activates basic leucine zipper transcription factors called AREB, which bind to ABRE element to induce the stress-responsive gene *RD29A*. Transcription factors such as DREB2A and DREB2B transactivate the DRE *cis*-element of osmotic stress genes and thereby are involved in maintaining the osmotic equilibrium of the cell. Some genes such as RD22 lack the typical CRT/DRE elements in their promoter suggesting their regulation by some other mechanism. The MYC/MYB transcription factors, RD22BP1 and AtMYB2, could bind MYCRS and MYBRS elements, respectively, and help in the activation of *RD22* gene [164, 165]. Overall, these transcription factors may also crosstalk with each other for their maximal response to stress tolerance.

4.9 Conclusions

In spite of the substantial efforts put in to unravel plant salt tolerance mechanisms, our understanding of the underlying molecular basis of salt tolerance is not clear yet. Though, recent advances in genetic analysis of *Arabidopsis* mutants defective in salt tolerance, and molecular cloning of these loci, have given us some insight into salt stress signaling and plant salt tolerance. Future research should be directed at (1) the identification of molecules connecting pathways and the key components of each pathway and (2) the characterization of individual genes and assessment of their contribution to salt stress tolerance, which will facilitate to engineer agronomically important salt-tolerant crop varieties.

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References

1 Chen, T.H.H. and Murata, N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.*, **5**, 250–257.
2 Zhu, J.K. (2001) Plant salt tolerance. *Trends Plant Sci.*, **6**, 66–71.
3 FAO (2000) Global network on integrated soil management for sustainable use of salt-affected soils http://www.fao.org/ag/AGL/agll/spush/intro.html.
4 Hasegawa, P.M., Bressan, R.A., Zhu, J.K., and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **51**, 463–499.
5 Parida, A.K. and Das, A.B. (2005) Salt tolerance and salinity effects on plants: a review. *Ecotoxicol. Environ. Safety*, **60**, 324–349.
6 Ashraf, M. (2004) Impact evaluation of water resources development in the common areas of small dams. Pakistan Council of Research in Water Resources, Research Report 5.
7 Tester, M. and Davenport, R. (2003) Na^+ tolerance and Na^+ transport in higher plants. *Ann. Bot.*, **91**, 503–527.
8 Flowers, T.J. (2004) Improving crop salt tolerance. *J. Exp. Bot.*, **55**, 307–319.
9 Munns, R. (2002) Comparative physiology of salt and water stress. *Plant Cell Environ.*, **25**, 239–250.
10 Munns, R. (2005) Genes and salt tolerance: bringing them together. *New Phytol.*, **167**, 645–663.
11 Munns, R., Cramer, G.R., and Ball, M.C. (1999) Interactions between rising CO_2, soil salinity and plant growth, in *Carbon Dioxide and Environmental Stress* (eds Y. Luo and H.A. Mooney), Academic Press, London, pp. 139–167.
12 Chinnusamy, V. and Zhu, J.K. (2003) Plant salt tolerance, in *Plant Responses to Abiotic Stress: Topics in Current Genetics*, vol. **4** (eds H. Hirt and

K. Shinozaki), Springer, Berlin, pp. 241–270.
13 Ashraf, M. and Foolad, M.R. (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.*, **59**, 206–216.
14 Tanji, K.K. (2002) Salinity in the soil environment, in *Salinity: Environment–Plant–Molecules* (eds A. Läuchli and U. Lüttge), Kluwer Academic Publishers, Dordrecht, pp. 21–51.
15 Läuchli, A. and Grattan, S.R. (2007) Plant growth and development under salinity stress, in *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops* (eds M.A. Jenks, P.M. Hasegawa, and S.M. Jain), Springer, Dordrecht, the Netherlands, pp. 285–315.
16 Flowers, T.J. and Flowers, S.A.M. (2005) Why does salinity pose such a difficult problem for plant breeders? *Agric. Water Manage.*, **78**, 15–24.
17 Carpici, E.B., Celika, N., Bayrama, G., and Asik, B.B. (2010) The effects of salt stress on the growth, biochemical parameter and mineral element content of some maize (*Zea mays* L.) cultivars. *Afr. J. Biotechnol.*, **9** (41), 6937–6942.
18 Smirnoff, N. (1998) Plant resistance to environmental stress. *Curr. Opin. Biotech.*, **9**, 214–219.
19 Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.*, **7**, 405–410.
20 Del Rio, L.A., Corpas, F.J., Sandalio, L.M., Palma, J.M., and Barroso, J.B. (2003) Plant peroxisomes, reactive oxygen metabolism and nitric oxide. *IUBMB Life*, **55**, 71–81.
21 Zhifang, G. and Loescher, W.H. (2003) Expression of a celery mannose 6-reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer. *Plant Cell Environ.*, **26**, 275–283.
22 Khan, M.H. and Panda, S.K. (2008) Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. *Acta Physyol. Plant*, **30**, 91–89.
23 Ashraf, M. and Haris, P.J.S. (2004) Pontential biochemical indicators of salinity tolerance in plants. *Plant Sci.*, **166**, 3–16.
24 Katerji, N., van Hoorn, J.W., Hamdy, A., and Mastrorilli, M. (2004) Comparison of corn yield response to plant water stress caused by salinity and by drought. *Agric. Water Manage.*, **65**, 95–101.
25 Arzani, A. (2008) Improving salinity tolerance in crop plants: a biotechnological view. *In Vitro Cell. Dev. Biol. Plant*, **44**, 373–383.
26 Dell'Aquila, A. (2000) Effect of combined salt and heat treatments on germination and heat-shock protein synthesis in lentil seeds. *Biol. Plantarum (Prague)*, **43**, 591–594.
27 Turkmen, O., Sensoy, S., Erdal, I., and Kabay, T. (2002) Effect of calcium on the emergence and seedling of tomatoes grown in salty growing media conditions. Yüzüncü Yıl University. *J. Agric. Sci.*, **12**, 53–57.
28 Burman, U., Garg, B.K., and Kathju, S. (2003) Water relations, photosynthesis and nitrogen metabolism of Indian mustard (*Brassica juncea* Czern. & Coss.) grown under salt and water stress. *J. Plant Biol.*, **30**, 55–60.
29 Wilson, C., Liu, X., Lesch, S.M., and Suarez, D.L. (2006) Growth response of major USA cowpea cultivars II. Effect of salinity on leaf gas exchange. *Plant Sci.*, **170**, 1095–1101.
30 Yang, X.H. and Lu, C.M. (2005) Photosynthesis is improved by exogenous glycinebetaine in salt-stressed maize plants. *Physiol. Plant*, **124**, 343–352.
31 Everard, J.D., Gucci, R., Kann, S.C., Flore, J.A., and Loescher, W.H. (1994) Gas-exchange and carbon partitioning in the leaves of celery (*Apium graveolens* L.) at various levels of root-zone salinity. *Plant Physiol.*, **106**, 281–292.
32 Netondo, G.W., Onyango, J.C., and Beck, E. (2004) Sorghum and salinity II. Gas exchange and chlorophyll fluorescence of sorghum under salt stress. *Crop Sci.*, **44**, 806–811.

33 Willmer, C.M. (1983) *Stomata*, Longman, London.

34 Mansour, M.M., Salama, F.G., Ali, M., and Abou Hadid, A.F. (2005) Cell and plant responses to NaCl in *Zea mays* L. cultivars differing in salt tolerance. *Gen. Appl. Plant Physiol.*, **31** (1–2), 29–41.

35 Gama, P.B., Inanaga, S., Tanaka, K., and Nakazawa, R. (2007) Physiological response of common bean (*Phaseolus vulg.*L.) seedlings to salinity stress. *Afri. J. Biotechnol.*, **6** (2), 79–88.

36 Kaymakanova1, M. and Stoeva1, N. (2008) Physiological reaction of bean plants (*Phaseolus vulg.* L.) to salt stress. *Gen. Appl. Plant Physiol.*, **34** (3–4), 177–188.

37 Hernandez, J.A., Mullineaux, P., and Sevilla, F. (2000) Tolerance of pea (*Pisum sativum L.*) to long term stress is associated with induction of antioxidant defences. *Plant Cell Environ.*, **23**, 853–862.

38 Demiral, T. and Turkan, I. (2005) Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. *Environ. Exp. Bot.*, **53**, 247–257.

39 Mandhania, S., Madan, S., and Sawhney, V. (2006) Antioxidant defense mechanism under salt stress in wheat seedlings. *Biol. Plant*, **227**, 227–231.

40 Turan, M.A., Türkmen, N., and Taban, N. (2007a) Effect of NaCl on stomatal resistance and proline, chlorophyll, Na, Cl and K concentrations of lentil plants. *J. Agron.*, **6**, 378–381.

41 Munns, R. (1993) Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. *Plant Cell Environ.*, **16**, 15–24.

42 Yousif, H.Y., Bingham, F.T., and Yermason, D.M. (1972) Growth, mineral composition, and seed oil of sesame (*Sesamum indicum* L.) as affected by NaCl. *Soil Sci. Soc. Am. Proc.*, **36**, 450–453.

43 Cordovilla, M.P., Ocana, A., Ligero, F., and Lluch, C. (1995) Salinity effects on growth analysis and nutrient composition in four grain legumes rhizobium symbiosis. *J. Plant Nutr.*, **18**, 1595–1609.

44 Lewis, O.A.M. (1986) The processing of inorganic nitrogen by the plant, in *Plants and Nitrogen* (ed. E. Arnold), Butterworth, London, England, pp. 21–41.

45 Wehrmann, I. and Hahndel, R. (1984) Relationship between N and Cl nutrition and NO3 content of vegetables. Proceedings of the VI International Colloquium for the Optimization of Plant Nutrition, 2, Montpellier, France, pp. 679–685.

46 Francois, L.E. and Mass, E.V. (1994) Crop response and management on salt affected soils, in *Hand Book of Plant and Crop Stress* (ed. M. Pessarakhli), Marcel Dekker, New York, NY, pp. 149–181.

47 Bartels, D. and Sunkar, R. (2005) Drought and salt tolerance in plants. *Crit. Rev. Plant Sci.*, **24**, 23–58.

48 Serrano, R. (1996) Salt tolerance in plants and microorganisms: toxicity targets and defense responses. *Int. Rev. Cytol.*, **165**, 1–52.

49 Apse, M.P. and Blumwald, E. (2002) Engineering salt tolerance in plants. *Curr. Opin. Biotechnol.*, **13**, 146–150.

50 Zhu, J.K. (2003) Regulation of ion homeostasis under salt stress. *Curr. Opin. Plant Biol.*, **6**, 441–445.

51 Ashraf, M.Y., Ashraf, M., and Sarwar, G. (2005) Physiological approaches to improving plant salt tolerance, in *Crops: Growth, Quality and Biotechnology* (ed. D. Ramdane), WFL Publisher, Helsinki, pp. 1206–1227.

52 Ashraf, F.M., Hameed, M., Arshad, M., Ashraf, M.Y., and Akhtar, K. (2006) Salt tolerance of some potential forage grasses from Cholistan desert of Pakistan, in *Ecophysiology of High Salinity Tolerant Plants* (eds M.A. Khan and D.J. Weber), Springer, The Netherlands, pp. 31–54.

53 Munns, R., James, R.A., and Läuchli, A. (2006) Approaches to increasing the salt tolerance of wheat and other cereals. *J. Exp. Bot.*, **57**, 1025–1043.

54 Karmoker, J.L., Farhana, S., and Rashid, P. (2008) Effects of salinity on ion

accumulation in maize (*Zea mays* L. cv. Bari–7). *Bangladesh J. Bot.*, **37**, 203–205.

55 Munns, R. and James, R.A. (2003) Screening methods for salinity tolerance: a case study with tetraploid wheat. *Plant Soil*, **253**, 201–218.

56 Song, J., Feng, G., and Zhang, F. (2006) Salinity and temperature effect on three salt resistant euhalophytes, *Halostachys capsica*, *Kalidium foliatum* and *Halocnemum strobilaceum*. *Plant Sci.*, **279**, 201–207.

57 Hameed, M., Ashraf, M., Ahmad, M.S.A., and Naz, N. (2010) Structural and functional adaptations in plants for salinity tolerance, in *Plant Adaptation and Phytoremediation* (eds M. Ashraf *et al.*.), Springer Science + Business Media, pp. 151–170.

58 Ball F M.C. (1988) Salinity tolerance in the mangroves, *Aegiceras corniculatum* and *Avicennia marina*. I. Water use in relation to growth, carbon partitioning and salt balance. *Aust. J. Plant Physiol.*, **15**, 447–464.

59 Amtmann, A. and Sanders, D. (1999) Mechanisms of Na^+ uptake by plant cells. *Adv. Bot. Res.*, **29**, 76–112.

60 Blumwald, E. and Gelli, A. (1997) Secondary inorganic ion transport in plant vacuoles. *Adv. Bot. Res.*, **25**, 401–407.

61 Tal, M. (1984) Physiological genetics of salt resistance in higher plants: studies on the level of the whole plant and isolated organs, tissues and cells, in *Salinity Tolerance in Plants: Strategies for Crop Improvement* (eds R.C. Staples and G.H. Toenniessen), John Wiley & Sons, Inc., New York, pp. 301–334.

62 Claussen F M., Luthen, H., Blatt, M., and Bottger, M. (1997) Auxin induced growth and its linkage to potassium channels. *Planta*, **201**, 227–234.

63 Yeo, A.R. (1998) Molecular biology of salt tolerance in the context of whole-plant physiology. *J. Exp. Bot.*, **49**, 915–29.

64 Kaiser, W.M. (1979) Reversible inhibition of the Calvin cycle and activation of oxidative pentose phosphate cycle in isolated chloroplast by hydrogen peroxide. *Planta*, **145**, 377–382.

65 Bohnert, H.J. and Jensen, R.G. (1996a) Metabolic engineering for increased salt tolerance: the next step. *Aust. J. Plant Physiol.*, **23**, 661–667.

66 Bohnert, H.J. and Jensen, R.G. (1996b) Strategies for engineering water-stress tolerance in plants. *Trends Biotech.*, **14**, 89–97.

67 Bohnert, H.J., Su, H., and Shen, B. (1999) Molecular mechanisms of salinity tolerance, in *Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants* (eds K. Shinozaki and K. Yamaguchi-Shinozaki), R.G. Landes Company, pp. 31–60.

68 Parvaiz, A. and Satyawati, S. (2008) Salt stress and phyto biochemical responses of plants – a review. *Plant Soil Environ.*, **54** (3), 89–99.

69 Yokoi, S., Quintero, F.J., Cubero, B., Ruiz, M.T., Bressan, R.A., Hasegawa, P.M., and Pardo, J.M. (2002) Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. *Plant J.*, **30**, 529–539.

70 Sairam, R.K. and Tyagi, A. (2004) Physiology and molecular biology of salinity stress tolerance in plants. *Curr. Sci.*, **86**, 407–421.

71 Shen, B., Jensen, R.G., and Bohnert, H.J. (1997a) Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. *Plant Physiol.*, **113**, 1177–1183.

72 Shen, B., Jensen, R.G., and Bohnert, H.J. (1997b) Mannitol protects against oxidation by hydroxyl radicals. *Plant Physiol.*, **115**, 527–532.

73 Orthen, B., Popp, M., and Smirnoff, N. (1994) Hydroxyl radical scavenging properties of cyclitols. *Proc. R. Soc. Edinb.*, **102**, 269–272.

74 Solomon, A., Beer, S., Waisel, Y. *et al.* (1994) Effects of NaCl on the carboxylating activity of Rubisco from *Tamarix jordanis* in the presence and absence of proline-related compatible solutes. *Plant Physiol.*, **90**, 198–204.

75 Galinski, E.A. (1993) Compatible solutes of halophilic eubacteria: molecular principles, water–solute interaction, stress protection. *Experientia*, **49**, 487–496.

76 Papageorgiou, G. and Murata, N. (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. *Photosyn. Res.*, **44**, 243–252.

77 Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.*, **53**, 247–273.

78 Xiong, L., Ishitani, M., Lee, H., and Zhu, J.K. (2001a) The *Arabidopsis LOS5/ABA3* locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. *Plant Cell*, **13**, 2063–2083.

79 Sheveleva, E., Chmara, W., Bohnert, H.J., and Jensen, R.G. (1997) Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. *Plant Physiol.*, **115**, 1211–1219.

80 Holmstrom, K.O., Somersalo, S., Mandal, A., Palva, T.E., and Welin, B. (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. *J. Exp. Bot.*, **51**, 177–185.

81 Kishitani, S., Takanami, T., Suzuki, M., Oikawa, M., Yokoi, S., Ishitani, M., Alvarez-Nakase, A.M., Takabe, T., and Takabe, T. (2000) Compatibility of glycine betaine in rice plants: evaluation using transgenic rice plants with a gene for peroxisomal betaine aldehyde dehydrogenase from barley. *Plant Cell Environ.*, **23**, 107–114.

82 Prasad, K.V.S.K., Sharmila, P., Kumar, P.A., and Saradhi, P.P. (2000) Transformation of *Brassica juncea* (L.) Czern with bacterial codA gene enhances its tolerance to salt stress. *Mol. Breed.*, **6**, 489–499.

83 Zhu, J.K., Liu, J., and Xiong, L. (1998) Genetic analysis of salt tolerance in *Arabidopsis thaliana*: evidence of a critical role for potassium nutrition. *Plant Cell*, **10**, 1181–1192.

84 Hong, Z., Lakkineni, K., Zhang, Z., and Verma, D.P.S. (2000) Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiol.*, **122**, 1129–1136.

85 Abd-El Baki, G.K., Siefritz, F., Man, H.M., Weiner, H., Kaldenhoff, R., and Kaiser, W. (2000) Nitrate reductase in *Zea mays* L. under salinity. *Plant Cell Environ.*, **23**, 515–521.

86 Cram, W.J. (1976) Negative feedback regulation of transport in cells. The maintenance of turgor, volume and nutrient supply, in *Encyclopaedia of Plant Physiology, New Series*, vol. 2 (eds U. Luttge and M.G. Pitman), Springer, Berlin.

87 Murakeozy, E.P., Nagy, Z., Duhaze, C., Bouchereau, A., and Tuba, Z. (2003) Seasonal changes in the levels of compatible osmolytes in three halophytic species of inland saline vegetation in Hungary. *J. Plant Physiol.*, **160**, 395–401.

88 Parida, A.K., Das, A.B., and Das, P. (2002) NaCl stress causes changes in photosynthetic pigments, proteins and other metabolic components in the leaves of a true mangrove, *Bruguiera parviflora*, in hydroponic cultures. *J. Plant Biol.*, **45**, 28–36.

89 Khavarinejad, R.A. and Mostofi, Y. (1998) Effects of NaCl on photosynthetic pigments, saccharides, and chloroplast ultrastructure in leaves of tomato cultivars. *Photosynthetica*, **35**, 151–154.

90 Ashraf, M. and Tufail, M. (1995) Variation in salinity tolerance in sunflower (*Helianthus annuus* L.). *J. Agron. Crop Sci.*, **174**, 351–362.

91 Ashraf, M. and Fatima, H. (1995) Responses of some salt tolerant and salt sensitive lines of safflower (*Carthamus tinctorius* L.). *Acta Physiol. Plant*, **17**, 61–71.

92 Ashraf, M. (1994) Organic substances responsible for salt tolerance in *Eruca sativa*. *Biol. Plant.*, **36**, 255–259.

93 Hounsa, C.G., Brandt, E.V., Thevelein, J., Hohmann, S., and Prior, B.A. (1998)

Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. *Microbiology*, **144**, 671–680.

94 Goddijn, O.J.M. and van Dun, K. (1999) Trehalose metabolism in plants. *TIBS*, **4**, 315–319.

95 Singer, M.A. and Lindquist, S. (1998) Multiple effects of trehalose on protein folding *in vitro* and *in vivo*. *Mol. Cell*, **1**, 639–648.

96 Yamada, T., Takatsu, Y., Manabe, T., Kasumi, M., and Marubashi, W. (2003) Suppressive effect of trehalose on apoptotic cell death leading to petal senescence in ethylene-insensitive flowers of gladiolus. *Plant Sci.*, **164**, 213–221.

97 Vogel, G., Aeschbacher, R.A., Muller, J., Boller, T., and Wiemken, A. (1998) Trehalose-6-phosphate phosphatases from *Arabidopsis thaliana*: identification by functional complementation of the yeast tps2 mutant. *Plant J.*, **13**, 673–683.

98 Bohnert, H.J. and Shen, B. (1999) Transformation and compatible solutes. *Sci. Hortic.*, **78**, 237–260.

99 Nelson, D.E., Koukoumanos, M., and Bohnert, H.J. (1999) Myo-inositol-dependent sodium uptake in ice plant. *Plant Physiol.*, **119**, 165–172.

100 Halliwell, B., Grootveld, M., and Gutteridge, J.M.C. (1988) Methods for the measurement of hydroxyl radicals in biochemical systems: deoxyribose degradation and aromatic hydroxylation. *Methods Biochem. Anal.*, **33**, 59–90.

101 Moran, J.F., Becana, M., Iturbe-Ormaetxe, I., Frechilla, S., Klucas, R.V., and Aparicio-Tejo, P. (1994) Drought induces oxidative stress in pea plants. *Planta*, **194**, 346–352.

102 Bohnert, H.J., Nelson, D.E., and Jensen, R.G. (1995) Adaptations to environmental stresses. *Plant Cell*, **7**, 1099–1111.

103 Tarcynski, M.C., Jensen, R.G., and Bohnert, H.J. (1992) Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science*, **259**, 508–510.

104 Binzel, M.L., Hess, F.D., Bressan, R.A., and Hasegawa, P.M. (1988) Intracellular compartmentation of ions in salt adapted tobacco cells. *Plant Physiol.*, **86**, 607–614.

105 Su, J., Chen, P.L., and Wu, R. (1999) Transgene expression of mannitol-1-phosphate dehydrogenase enhanced the salt stress tolerance of the transgenic rice seedlings. *Sci. Agric. Sin.*, **32**, 101–103.

106 Ford, C.W. (1984) Accumulation of low molecular weight solutes in water stressed tropical legumes. *Phytochemistry*, **23**, 1007–1015.

107 Gorham, J., Hughes, L.L., and Wyn Jones, R.G. (1981) Low-molecular-weight carbohydrates in some salt-stressed plants. *Physiol. Plant*, **53**, 27–33.

108 Gorham, J., McDonnel, E., and Wyn Jones, R.G. (1984) Pinitol and other solutes in salt-stressed *Sesbania aculeata, Z. Pflanzenphysiol. Bd.*, **114**, 173–178.

109 Fougere, F., Le Rudulier, D., and Streeter, J.G. (1991) Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago sativa* L.). *Plant Physiol.*, **96**, 1228–1236.

110 Loewus, F.A. and Dickinson, D.B. (1982) Cyclitols, in *Encyclopedia of Plant Physiology: Plant Carbohydrates 1. Intracellular Carbohydrates*, vol. **13A** (eds F.A. Loewus and W. Tanner), Springer, Berlin, pp. 43–76.

111 Vernon, D.M. and Bohnert, H.J. (1992) A novel methyl transferase induced by osmotic stress in the facultative halophytes *Mesembryanthemum crystallinum*. *EMBO J.*, **11**, 2077–2085.

112 Vernon, D., Tarcynski, M.C., Jensen, R.G., and Bohnert, H.J. (1993) Cyclitol production in transgenic tolerance. *Plant J.*, **4**, 199–205.

113 Vinocur, B. and Altman, A. (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr. Opin. Biotech.*, **16**, 123–132.

114 Ketchum, R.E.B., Warren, R.C., Klima, L.J., Lopez-Gutierrez, F., and Nabors, M.W. (1991) The mechanism and regulation of proline accumulation in

suspension cultures of the halophytic grass *Distichlis spicata* L. *J. Plant Physiol.*, **137**, 368–374.

115 Kishor, P.B.K., Hong, Z., Miao, G.H., Hu, C.A.A., and Verma, D.P.S. (1995) Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.*, **108**, 1387–1394.

116 Yan, H., Gong, L.Z., Zhao, C.Y., and Guo, W.Y. (2000) Effects of exogenous proline on the physiology of soyabean plantlets regenerated from embryos *in vitro* and on the ultrastructure of their mitochondria under NaCl stress. *Soybean Sci.*, **19**, 314–319.

117 Ueda, A., Yamamoto-Yamane, Y., and Takabe., T. (2007) Salt stress enhances proline utilization in the apical region of barley roots. *Biochem. Biophys. Res. Commun.*, **355**, 61–66.

118 Ueda, A., Shi, A., Shimada, T., Miyake, H., and Takabe., T. (2008) Altered expression of barley proline transporter causes different growth responses in *Arabidopsis*. *Planta*, **227**, 277–286.

119 Petrusa, L.M. and Winicov, I. (1997) Proline status in salt tolerant and salt sensitive alfalfa cell lines and plants in response to NaCl. *Plant Physiol. Biochem.*, **35**, 303–310.

120 Ahmad, I., Wainwright, S.N., and Stewart, G.R. (1981) The solute and water relations of *Agrostis stolonifera* ecotypes differing in their salt tolerance. *New Phytol.*, **87**, 615–629.

121 Jain, S., Nainawatee, H.S., Jain, R.K., and Chowdhury, J.B. (1991) Proline status of genetically stable salt-tolerant *Brassica juncea* L. somaclones and their parent cv. 'Parkash.' *Plant Cell Rep.*, **9**, 684–687.

122 Kirti, P.B., Hadi, S., and Chopra, V.L. (1991) Seed transmission of salt tolerance in regenerants of *Brassica juncea* selected *in vitro*. *Crucif. Newsl.*, **85**, 14–15.

123 Roosens, N., Hal Bitar, F., Loenders, K., Angenon, G., and Jacobs, M. (2002) Overexpression of ornithine-δ-aminotransferase increases proline biosynthesis and confers osmotolerance in transgenic plants. *Mol. Breed.*, **9**, 73–80.

124 Mani, S., van de Cotte, B., Van Montagu, M., and Verbruggen, N. (2002) Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in *Arabidopsis*. *Plant Physiol.*, **128**, 73–83.

125 Rhodes, D. and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.*, **44**, 357–384.

126 Weretilnyk, E.A., Bednarek, S., McCue, K.F., Rhodes, D., and Hanson, A.D. (1989) Comparative biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. *Planta*, **178**, 342–352.

127 Yang, T. and Poovaiah, B.W. (2003) Calcium/calmodulin-mediated signal network in plants. *Trends Plant Sci.*, **8**, 505–512.

128 Murata, N., Mohanty, P.S., Hayashi, H., and Papageorgiou, G.C. (1992) Glycinebetaine stabilizes the association of extrinsic proteins with the photosynthetic oxygen evolving complex. *FEBS Lett.*, **296**, 187–189.

129 Agastian, P., Kingsley, S.J., and Vivekanandan, M. (2000) Effect of salinity on photosynthesis and biochemical characteristics in mulberry genotypes. *Photosynthetica*, **38**, 287–290.

130 Saneoka, H., Nagasaka, C., Hahn, D.T., Yang, W.J., Premachandra, G.S., Joly, R.J., and Rhodes, D. (1995) Salt tolerance of glycinebetaine-deficient and -containing maize lines. *Plant Physiol.*, **107**, 631–638.

131 Hayashi, H., Alia, Mustardy, L., Deshnium, P., Ida, M., and Murata, N. (1997) Transformation of *Arabidopsis thaliana* with the codA gene for choline oxidase: accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. *Plant J.*, **12**, 133–142.

132 Nuccio, M.L., Rhodes, D., McNeil, S.D., and Hanson, A.D. (1999) Metabolic engineering of plants for osmotic stress

resistance. *Curr. Opin. Plant Biol.*, **2**, 128–134.

133 Huang, J., Hirji, R., Adam, L., Rozwadowski, K.L., Hammerlindl, J.K., Keller, W.A., and Selvaraj, G. (2000) Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiol.*, **122**, 747–756.

134 Rontein, D., Basset, G., and Hanson, A.D. (2002) Metabolic engineering of osmoprotectant accumulation in plants. *Metab. Eng.*, **4**, 49–56.

135 Lutts, S. (2000) Exogenous glycine betaine reduces sodium accumulation in salt-stressed rice plants. *Int. Rice Res. Notes*, **25**, 39–40.

136 Makela, P., Jokinen, K., Kontturi, M., Peltonen-Sainio, P., Pehu, E., and Somersalo, S. (1998) Foliar application of glycine betaine – a novel product from sugar beet, as an approach to increase tomato yield. *Ind. Crops Prod.*, **7**, 139–148.

137 Ingram, J. and Bartels, D. (1996) The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 377–403.

138 Singh, N.K., Bracken, C.A., Hasegawa, P.M., Handa, A.K., Buckel, S., Hermodson, M.A., Pfankoch, F., Regnier, F.E., and Bressan, R.A. (1987) Characterization of osmotin. A thaumatin-like protein associated with osmotic adjustment in plant cells. *Plant Physiol.*, **85**, 529–536.

139 Pareek, A., Singla, S.L., and Grover, A. (1997) Salt responsive proteins/genes in crop plants, in *Strategies for Improving Salt Tolerance in Higher Plants* (eds P.K. Jaiwal, R.P. Singh, and A. Gulati), Oxford and IBH Publication Co., New Delhi, pp. 365–391.

140 Thomas, J.C. and Bohnert, H.J. (1993) Salt stress perception and plant growth regulators in the halophyte *Mesembryanthemum crystallinum*. *Plant Physiol.*, **103**, 1299–1304.

141 Hurkman, W.J., Rao, H.P., and Tanaka, C.K. (1991) Germin-like polypeptides increase in barley roots during salt stress. *Plant Physiol.*, **97**, 366–374.

142 Lopez, F., Vansuyt, G., Fourcroy, P., and Case-Delbart, F. (1994) Accumulation of a 22-kDa protein and its mRNA in the leaves of *Raphanus sativus* in response to salt stress or water stress. *Physiol. Plant*, **91**, 605–614.

143 Uma, S., Prasad, T.G., and Kumar, M.U. (1995) Genetic variability in recovery growth and synthesis of stress proteins in response to polyethylene glycol and salt stress in finger millet. *Ann. Bot.*, **76**, 43–49.

144 Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.D., and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.*, **110**, 249–257.

145 Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Molecular response to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr. Opin. Plant Biol.*, **3**, 217–223.

146 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotech.*, **17**, 287–291.

147 Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D., and Thomashow, M.F. (2000) Overexpression of the *Arabidopsis CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.*, **124**, 1854–1865.

148 Xiong, L., Schumaker, K.S., and Zhu, J.K. (2002b) Cell signaling for cold, drought, and salt stresses. *Plant Cell*, **14**, S165–S183.

149 Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant. Biol.*, **55**, 373–399.

150 Foyer, C.H., Descourvieres, P., and Kunert, K.J. (1994) Protection against oxygen radicals, an important defense mechanism studied in transgenic plants. *Plant Cell Environ.*, **17**, 507–523.

151 Halliwell, B. and Gutteridge, J.M.C. (1999) *Free Radicals in Biology and*

Medicine, 3rd edn, Oxford University Press, New York.

152 Shalata, A., Mittova, V., Volokita, M., Guy, M., and Tal, M. (2001) Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt-dependent oxidative stress: the root antioxidative system. *Physiol. Plant*, **112**, 487–494.

153 Kranner, I., Beckett, R.P., Wornik, S., Zorn, M., and Pfeifhofer, H.W. (2002) Revival of resurrection plant correlates with its antioxidant status. *Plant J.*, **31**, 13–24.

154 Sairam, R.K. and Srivastava, G.C. (2002) Changes in antioxidant activity in subcellular fractions of tolerant and susceptible wheat genotypes in response to long-term salt stress. *Plant Sci.*, **162**, 897–904.

155 Gossett, D.R., Banks, S.W., Millhollon, E.P., and Lucas, M.C. (1996) Antioxidant response to NaCl stress in a control and a NaCl-tolerant cotton line grown in the presence of paraquat, buthionine sulfoxime, and exogenous glutathione. *Plant Physiol.*, **112**, 803–809.

156 Shalata, A. and Tal, M. (1998) The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. *Physiol. Plant*, **104**, 169–174.

157 Mittova, V., Guy, M., Tal, M., and Volokita, M. (2002) Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt-dependent oxidative stress: increased activities of antioxidant enzymes in root plastids. *Free Radic. Res.*, **36**, 195–202.

158 Allen, R. (1995) Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol.*, **107**, 1049–1054.

159 Roxas, V.P., Smith Jr, R.K., Allen, E.R., and Allen, R.D. (1997) Overexpression of glutathione-*S*-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nat. Biotechnol.*, **15**, 988–991.

160 Roxas, V.P., Sundus, A.L., Garrett, D.K., Mahan, J.R., and Allen, R.D. (2000) Stress tolerance in transgenic tobacco seedlings that over express glutathione-*S*-transferase/glutathione peroxidase. *Plant Cell Physiol.*, **41**, 1229–1234.

161 Tanaka, Y. *et al.* (1999) Salt tolerance of transgenic rice over-expressing yeast mitochondrial Mn-SOD in chloroplasts. *Plant Sci.*, **148**, 131–138.

162 Knight, H., Trewavas, A.J., and Knight, M.R. (1997) Calcium signaling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J.*, **12**, 1067–1078.

163 Sanders, D., Brownlee, C., and Harper, J.F. (1999) Communicating with calcium. *Plant Cell*, **11**, 691–706.

164 Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.*, **444**, 139–158.

165 Tuteja, N. (2007) Mechanisms of high salinity tolerance in plants. *Meth. Enzymol.*, **428**, 419–438.

166 Tuteja, N. and Mahajan, S. (2007) Further characterization of calcineurin B-like protein and its interacting partner CBL-interacting protein kinase from *Pisum sativum*. *Plant Sig. Behav.*, **2**, 358–361.

167 DeWald, D.B., Torabinejad, J., Jones, C.A., Shope, J.C., Cangelosi, A.R., Thompson, J.E., Prestwich, G.D., and Hama, H. (2001) Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate correlates with calcium mobilization in salt-stressed *Arabidopsis*. *Plant Physiol.*, **126**, 759–769.

168 Takahashi, S., Katagiri, T., Hirayama, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2001) Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in *Arabidopsis* cell culture. *Plant Cell Physiol.*, **42**, 214–222.

169 Pical, C., Westergren, T., Dove, S.K., Larsson, C., and Sommarin, M. (1999) Salinity and hyperosmotic stress induce rapid increases in phosphatidylinositol 4,5-bisphosphate, diacylglycerol pyrophosphate, and phosphatidylcholine in *Arabidopsis thaliana* cells. *J. Biol. Chem.*, **274**, 38232–38240.

170 Hirayama, T., Ohto, C., Mizoguchi, T., and Shinozaki, K. (1995) A gene encoding a phosphatidylinositol-specific

phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, **92**, 3903–3907.

171 Xiong, L., Lee, B.-H., Ishitani, M., Lee, H., Zhang, C., and Zhu, J.-K. (2001b) *FIERY1* encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in *Arabidopsis*. *Genes Dev.*, **15**, 1971–1984.

172 Quintero, F.J., Garciadeblas, B., and Rodriguez-Navarro, A. (1996) The *SAL1* gene of *Arabidopsis*, encoding an enzyme with 3′(2′),5′-bisphosphate nucleotide and inositol polyphosphate 1-phosphatase activities, increases salt tolerance in yeast. *Plant Cell*, **8**, 529–537.

173 Wu, Y., Lei, D., and Zhu, J.K. (1996) *SOS1*, a genetic locus essential for salt tolerance and potassium acquisition. *Plant Cell*, **8**, 617–627.

174 Mahajan, S., Pandey, G., and Tuteja, N. (2008) Calcium- and salt-stress signaling in plants: shedding light on SOS pathway. *Arch. Biochem. Biophys.*, **471** (2), 146–158.

175 Quintero, F.J., Ohta, M., Shi, H., Zhu, J.K., and Pardo, J.M. (2002) Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for Naþ homeostasis. *Proc. Natl. Acad. Sci. USA.*, **99**, 9061–9066.

176 Apse, M.P., Aharon, G.S., Snedden, W.A., and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Naþ=Hþ antiport in *Arabidopsis*. *Science*, **285**, 1256–1258.

177 Zepeda-Jazo, I., Shabala, S., Chen, Z., and Pottosin, I.I. (2008) Na^+–K^+ transport in roots under salt stress. *Plant Sig. Behav.*, **3**, 401–403.

178 Chinnusamy, V., Schumaker, K., and Zhu, J.-K. (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signaling in plants. *J. Exp. Bot.*, **55**, 225–236.

179 Jia, W., Wang, Y., Zhang, S., and Zhang, J. (2002) Salt-stress-induced ABA accumulation is more sensitively triggered in roots than in shoots. *J. Exp. Bot.*, **53**, 2201–2206.

180 Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997) Gene expression and signal transduction in water-stress response. *Plant Physiol.*, **115**, 327–334.

181 Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M., and Waner, D. (2001) Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **52**, 627–658.

182 Zhu, J., Gong, Z., Zhang, C., Song, C.-P., Damsz, B., Inan, G., Koiwa, H., Zhu, J.K., Hasegawa, P.M., and Bressan, R.A. (2002) OSM1/SYP61: a syntaxin protein in *Arabidopsis* controls abscisic acid-mediated and non-abscisic acid-mediated responses to abiotic stress. *Plant Cell*, **14**, 3009–3028.

183 Yamaguchi-Shinozaki, K. and Shinozaki, K. (1993) Characterization of the expression of a desiccation-responsive *rd*29 gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. *Mol. Gen. Genet.*, **236**, 331–340.

184 Sanan-Mishra, N., Phan, X.H., Sopory, S.K., and Tuteja, N. (2005) Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. *Proc. Natl Acad. Sci. USA.*, **102**, 509–514.

185 Mahajan, S., Sopory, S.K., and Tuteja, N. (2006b) Cloning and characterization of CBL-CIPK signaling components from a legume (*Pisum sativum*). *FEBS J.*, **273**, 907–925.

5
Cold and Abiotic Stress Signaling in Plants

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Plants, unlike animals, are not mobile, and therefore unable to avoid stressful environmental conditions. As a consequence, plants have developed a wide variety of protective and defensive mechanisms in order to survive adverse conditions. Abiotic stresses, such as low temperature, high salinity, drought, or osmotic stress, are common challenges to plant survival, and negatively affect plant growth and development. From the point of view of molecular biology and biochemistry, stress signals are, in most cases, perceived by cellular receptors sitting on the cell membranes. These receptors transmit their information through multiple intermediate components in the signal transduction pathways, in order to finally activate the effector proteins, which are involved in executing physiological changes inside plant cells. However, most abiotic stress signalings, such as low-temperature stress signaling, are not linear, but are interconnected with each other. A myriad of molecules and proteins, including ions, lipids, protein kinases, and transcription factors, participate in the stress signal transduction networks. Such complex signaling networks pose a huge challenge to scientists attempting to understand the molecular and biochemical mechanisms plants employ to survive life-threatening conditions. Nevertheless, with the development of a number of advanced technologies, such as DNA microarray for global mRNA profiling, LC/MS/MS mass spectrometry for global proteomic profiling, and an abundance of new functional genomics tools, significant progress has been made in illuminating the mechanisms of plant abiotic stress responses. This chapter summarizes the most recent discoveries about the components involved in plant abiotic stress, with an emphasis on cold stress responses, and focuses on elucidating the functions of these components and their contribution to cold signaling.

5.1
Introduction

Plants, as sessile organisms, are constantly exposed to a variety of unfavorable environmental conditions, and their ability to cope with such conditions is essential

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to their survival. A large number of temperate plants handle sudden chilling (<10 °C) and freezing (<0 °C) stress through a process known as cold acclimation, an acquired plant defensive response. This response involves transcriptional activation and expression of a large number of genes that function in signal transduction, transcriptional regulation, and synthesis of protective enzymes and metabolites. However, many tropical and subtropical plants, including important crop species such as rice, maize, tomato, cotton, and soybean lack basic tolerance to low or freezing temperatures, and do not have the ability to acclimate to cold temperatures; thus, they are subject to chilling and freezing damage. As a result, cold stress negatively affects many ecologically and economically important plant species, and can be an important limiting factor in plant growth and yield.

Cold acclimation has been extensively studied for the past 10–15 years, and significant progress has been made in understanding the underlying molecular mechanisms by which this process is regulated during plant cold and freezing responses [1–3]. Transcriptomic and metabolomic analyses have indicated that a dramatic reprogramming of gene expression and cellular metabolism occurs during cold acclimation, and it appears that a large number of signaling molecules and transcription factors play key roles in the regulation of this process [4–8].

In this chapter, the latest studies are summarized, emphasizing cold signaling perception, transduction, and transcriptional and posttranscriptional gene regulation. Owing to the power of genetics and most recently the development of functional genomics, most of the fundamental discoveries in plant cold stress response have been in *Arabidopsis*. Consequently, this chapter is mainly focused on the model plant *Arabidopsis thaliana*, while only briefly mentioning other plant species. Many of the signaling molecules and pathways discussed in this chapter are also involved in plant response to other abiotic stresses, such as salt, drought, and osmotic stresses. However, due to the extensive details of the comprehensive signaling pathways known for each of those stresses, mainly plant cold stress response is covered here.

5.2
Gene Expression and Regulation during Plant Cold Stress Response

Genome-wide transcriptome analyses have shown that as much as 10–20% of the genome displays changes at transcript levels following cold stress treatment [1, 9]. These substantial changes in gene expression result in a complete reprogramming of the cellular metabolism, physiology, and subsequent remodeling of plant growth and development. The genome-wide transcriptional responses to cold stress can be divided into two stages: the early and the late stage. Genes involved in the early-stage response mainly encode transcriptional factors, and genes involved in the late-stage cold stress response belong to a set of core cold-inducible genes. The late-stage cold-inducible gene set includes *COR* (cold regulated), *KIN* (cold induced), *LTI* (low-temperature induced), *RD* (responsive to dehydration), and *LEA* (late embryogenesis abundant), whose products are directly involved in stress protection and

maintenance of cellular homeostasis. The rapid transcriptional activation of a number of transcription factor genes is considered to be key to the subsequent activation of a vast number of genes involved in cellular metabolisms and stress protection. Among these transcription factors, the C-repeat-binding factors (*CBFs*), or dehydration-responsive element binding factors 1 (*DREB1s*), have been shown to be essential for plants to tolerate cold stresses [10, 11], although a number of other transcription factors are also indispensable.

5.2.1 The CBF/DREB-Dependent and Independent Pathways

The *CBF/DREB1* genes were originally identified in *Arabidopsis* through yeast one-hybrid screening, using the CRT (C-repeat)/DRE (dehydration response element) elements as the bait [10, 11]. The corresponding proteins, which belong to the plant-specific ERF/AP2 superfamily transcription factors, can activate the expression of a battery of downstream target genes, also called CBF/DREB regulon genes, by binding to CRT/DRE elements in the promoter regions of the target genes [12]. In *Arabidopsis*, there exist three *CBF/DREB1* genes, namely, *CBF1–3*, or *DREB1b*, *c*, and *a*, respectively. These three genes are organized in tandem array at the same chromosomal location. More importantly, it has been shown that the message levels of all three *CBF/DREB* genes are quickly (within 15 min) and specifically induced by cold stress [4, 5, 7, 10, 11]. The essential role of the *CBF/DREB1* gene during plant cold stress response was demonstrated through reverse genetic studies where the overexpression of the *CBF3/DREB1A* or *CBF2/DREB1C* in transgenic *Arabidopsis* plants resulted in the activation of *COR* gene expression even at the moderate temperature and significantly enhanced cold and freezing tolerance [13]. Although cold signaling pathways other than those mediated by *CBF/DREB1* do exist (discussed later), it has been shown that CBFs/DREBs play a major role in the regulation of the cellular transcriptome following cold stress.

The CBF/DREB1-like proteins are present in a variety of plant species, including not only the plants from temperate regions, such as *Arabidopsis*, barley, wheat, and *Brassica napus* [14, 15], which can cold-acclimate, but also the plants from tropical and subtropical regions, such as rice, maize, and tomato [15–18], which do not cold-acclimate, indicating that the CBF/DREB1 cold-responsive pathway is conserved among the plant kingdom. However, the higher cold and freezing sensitivity of crop plants clearly suggests that CBF/DREB1 is not sufficient and other components and/or pathways are needed to confer improved resistance to cold stress.

Through a genetic mutant screening, the *Arabidopsis Eskimo1* gene was identified as a negative regulator in plant cold stress response [19]. The loss-of-function *esk1* mutant accumulates higher levels of compatible osmolytes, such as proline, under normal growth conditions, is constitutively tolerant to freezing, and has much higher freezing tolerance (5.5 °C improvement) than wild-type plants. Transcriptome analysis showed that only 12 of the total 312 genes with altered mRNA expression were also present in the *CBF2* gene regulon, indicating that ESK1 mostly functions

independent of CBF/DREB1 [20]. The *ESK1* gene encodes a DUF231, which belongs to a large family of proteins with domain of unknown function [20]. It is important to note that the expression of the *ESK1* gene itself is not altered by cold stress [20], suggesting that posttranscriptional regulatory mechanisms might be involved in activation of the ESK1 protein. However, the molecular mechanism by which ESK1 regulates cold stress signaling pathways still remains elusive.

Other CBF/DREB-independent pathways include the HOS9-mediated pathway. HOS9 was identified through a genetic screening that employed the promoter of the cold-inducible *RD29A* gene fused to the luciferase reporter gene [21, 22]. Transgenic *Arabidopsis* plants containing the P_{RD29A}-LUC system were screened for mutants that have enhanced luciferase expression specifically following cold stress treatment. The *hos9* null mutants have defects in freezing resistance, regardless of whether they have been cold acclimated or not [22]. More intriguingly, the expression of a number of cold-responsive genes such as *RD29A* and *COR15* were much stronger than wild type [22]. From microarray data analysis of the *hos9* null mutant, it appears that the HOS9 regulon is distinct from the CBF regulon [22]. *HOS9* encodes a homeodomain transcription factor, and like *ESK1* gene, the mRNA expression of the HOS9 gene is constitutively high and not responsive to cold treatment [22], thus raising the possibility that this gene is also subject to posttranscriptional regulation.

Another important and well-characterized CBF/DREB1-independent, cold-responsive pathway is the ABA-mediated pathway. ABA is an important plant hormone involved in stomatal closure, seed maturation, and dormancy [23, 24]. The role of ABA in plant stress response, especially to dehydration/drought and osmotic stress, has been well recognized [3, 25]. However, its role in cold stress response remains somewhat inconclusive. It was shown that plants have increased levels of endogenous ABA following salt, drought, and cold stresses, although such increase after cold stress is not as pronounced as the other two stresses [26, 27]. In a study with mutants that were either ABA deficient (*aba1-1*) or ABA insensitive (*abi1-1*), it was shown that ABA was required for the cold acclimation process, and mutants that had blocked ABA biosynthesis and signaling lost the ability to cold acclimate, thus were hypersensitive to freezing stress [28].

In addition, it was found that the mRNA expression of many cold stress-responsive genes could also be induced by exogenous ABA treatment [29–31]. In a transcriptome analysis with *Arabidopsis* plants following 3 and 24 h cold stress treatment, both the ABRE (ABA-responsive element) and the CRT/DRE element were significantly enriched in the cluster of late-cold-responsive genes [4], suggesting that these two independent pathways might act synergistically in regulating the expression of cold-responsive genes. Transcription factors belonging to the class of bZIP transcription factor are called ARE proteins or ABFs and bind to the ABRE [32]. Most importantly, through a transient expression analysis using a protoplast system, it was found that transcription factors AREB1/ABF2, AREB4/ABF4, and ABF3 could directly activate the reporter gene expression by binding to the ABRE element, and the transcriptional activities were much more compromised in both the ABA-deficient *aba2* and the ABA-insensitive (*abi1*) mutants,

strongly suggesting that these transcription factors play crucial roles in the ABA signaling and stress responses [33].

5.2.2 Regulation of ICE1-CBF/DREB Gene Expression

5.2.2.1 Transcriptional Regulation

Although extensive knowledge has been accumulated about transcriptional events downstream of the CBF/DREB1 proteins, the regulatory and signaling events upstream of CBF/DREB1 proteins have just begun to be uncovered. Owing to the rapid and transient induction of mRNA expression of the *CBF/DREB1* genes following cold stress treatment, it was hypothesized that one or more constitutively expressed gene(s) may be required. The search for such genes resulted in the identification of *ICE1* from a genetic screening for inducers of the *CBF/DREB1* expression [34]. *ICE1* encodes a MYC-like bHLH transcription factor. The phenotype of the *ice1* null mutant plant includes loss of the inducible expression of the *CBF3/DREB1A* gene, and compromised ability in cold stress tolerance [34]. The constitutive expression of the *ICE1* gene at the mRNA level raised the question about how this gene is activated by cold stress. A number of potential serine/threonine phosphorylation sites were identified, suggesting posttranslational modification might play a role in regulating the activity of this protein; however, the exact roles of these phosphorylation sites have not been reported.

Although ICE1 regulates the expression of the *CBF3/DREB1A* gene, it does not have any effect on the other two *CBF/DREB1*s, regardless of the existence of a consensus recognition site for the bHLH transcription factors within the *CBF2/DREB1c* promoter [35]. This suggests that other MYC-like bHLH transcription factors might be involved in the regulation of this gene. In the search for other transcriptional mechanisms that regulate *CBF/DREB1* gene expression, promoters of the *CBF/DREB1* genes were rescanned and potential binding sites were revealed for both MYC- and MYB-like transcription factors [36].

The *Arabidopsis MYB15* gene, which belongs to the R2R3-MYB family of transcription factors, was then identified through microarray data analysis of genes whose expression was induced upon cold treatment [36]. More interestingly, microarray data also indicated that the expression of the *MYB15* gene was enhanced in the *ice1* mutant, while yeast two-hybrid analysis showed that MYB15 physically interacted with ICE1. This suggests that MYB15 is negatively regulated by ICE1, and likely functions in cold stress signaling through the ICE-CBF pathway. However, rather than acting as a transcription activator like ICE1, a transient expression assay showed that MYB15 acts as a transcription repressor and represses the expression of *CBF1–3/DREB1A–C*. Further supporting this data, it was found that the expression of the *CBF1–3/DREB1A–C* genes was enhanced in the *myb15* loss-of-function mutant, which in turn resulted in improved freezing tolerance.

Another negative regulator involved in the regulation of *CBF/DREB1* expression is ZAT12. The *Arabidopsis ZAT12* gene, which encodes a C2H2 zinc finger transcription

factor, was identified through microarray data analysis as one of the six transcription factor genes whose mRNA expression is upregulated within only 1 h of cold stress treatment [37]. Overexpression of the *ZAT12* gene in transgenic *Arabidopsis* plants results in small but reproducible improvement in freezing stress tolerance [37]. Consistent with such marginal improvement, the ZAT12 regulon is much smaller in comparison to the CBF regulon that comprises about 28% of the cold-inducible genes [37]. However, it is interesting to note that the expression of the *CBF1–3/DREB1A–C* genes is significantly reduced, although not completely abolished, in transgenic *Arabidopsis* plants overexpressing *ZAT12*. Moreover, there is a considerable overlap between the ZAT12 regulon and the CBF regulon, suggesting that CBFs/DREBs and ZAT12 might interact in coordinating the cold signaling pathways [37].

In addition to the different types of transcription factors that are involved in the regulation of *CBF/DREB1* expression, surprisingly, CBFs appear to be involved in the feedback regulation of their own genes [38]. A case in point: it was found that the expression of *CBF3/DREB1A* and *CBF1/DREB1B* was much stronger and more sustained in the *cbf2/dreb1b* mutant, and this mutant has higher resistance to freezing and other abiotic stresses.

5.2.2.2 **Posttranscriptional Regulation**

Posttranscriptional regulation of plant gene expression during cold stress response was relatively overlooked until recently when genes involved in mRNA processing, translocation, and stability were identified from a variety of molecular genetic studies. The transcriptional activation of a large number of genes following cold stress results in the accumulation of a large amount of nascent mRNAs inside the nucleus. These pre-mRNAs need to be properly processed and exported to the cytosol, where the mRNAs can be translated into functional proteins [39].

One of the genes involved in the splicing process of the cold-inducible *COR15* gene and possibly other genes is the *Arabidopsis STA1* (for *STABILIZED1*) [40]. The *STA1* gene was cloned via the same P_{RD29A}-LUC imaging system used to search for mutants with enhanced stability of the luciferase activity. It was found to encode a 102-kD nuclear protein similar both to the human U5 small ribonucleoprotein-associated protein and to the yeast pre-mRNA splicing factors Prp1p and Prp6p. The mRNA expression of the *STA1* gene is itself cold inducible, and plants harboring mutation in the *STA1* gene contain substantially higher levels of unspliced *COR15* mRNA than wild-type plants. They also displayed reduced chilling tolerance, as well as hypersensitivity to ABA and salt stress [40].

The nucleoporin protein, *AtNUP160*, and the DEAD-box RNA helicase encoded by the *LOS4* (low expression of osmotically responsive genes 4) gene were found to play essential roles in properly unwinding tight RNA secondary structures and transporting unwound RNAs from the nucleus to the cytoplasm [41–43]. While the *los4* mutant was identified through the same P_{RD29A}-LUC imaging system, the *atnup160* mutant was identified through a slightly different screening system that involved the promoter from the *CBF3/DREB1A* gene fused to luciferase. Both the *los4* and the *atnup160* mutants were hypersensitive to chilling and freezing stresses, and poly(A)-mRNA export from nucleus to cytoplasm was much lower under cold stress

conditions in both these mutants, compared to wild-type plants, suggesting that the defects in cold response of these mutants might be caused by the retention of cold-responsive mRNAs in the nucleus. Consistent with their possible role in RNA metabolism and transportation, both LOS4 and AtNUP160 proteins are enriched at the nuclear rim [41, 43].

The *Arabidopsis FIERY2 (FRY2)/CPL1* gene is another important factor worth mentioning. This gene is involved in coordinated pre-mRNA processing, functional mRNA formation, and mRNA export [25, 44]. *FIERY2 (FRY2)/CPL1* encodes a protein phosphatase that directly regulates the activity of RNA polymerase II by dephosphorylating the RNA polymerase II C-terminal domain (CTD). It has been shown that the phosphorylation states of the CTD domain directly correlate with the functional involvement of RNA polymerase II (RNAP II) in transcription, as RNAP II with a hypophosphorylated CTD (RNAP IIA) is included preferentially in the transcription preinitiation complex formed at the promoter, whereas RNAP II with a hyperphosphorylated CTD (RNAP IIO) is associated with elongation complexes [44]. The *fry2/cpl1* mutants were obtained through the P_{RD29A}-LUC imaging system and displayed more enhanced cold-responsive gene expression. It is worth noting that while the *fry2/cpl1* mutants showed a much more reduced freezing tolerance, the expression of *CBF/DREB1* genes was surprisingly higher in these mutants, suggesting that the FRY2/CPL1 protein functions independent of CBF/DREB1 [25].

5.2.2.3 Involvement of Small RNAs in the Plant Cold Stress Response

Plant small RNAs, including microRNA (miRNA) and short interfering RNA (siRNA), are a class of highly conserved, nonprotein-coding RNAs ranging in size between 20 and 24 nucleotides. Generally considered as transcriptional repressors, microRNAs and siRNA exert their functions mainly through mRNA cleavage or translational repression of complementary target mRNAs [45–51]. Interestingly, it has been predicted that one of the major classes of target genes for plant small RNAs is transcriptional regulator, and translational repression of these transcription regulators consequently leads to the inhibition of mRNA expression of a large number of downstream genes [52, 53]. In accordance with this prediction, plant small RNAs, especially miRNAs, have been found to be involved both in a number of developmental processes, such as leaf and flowering development, and in other cellular processes, such as auxin signaling [45, 47, 54, 55]. Only recently have researchers discovered that they also play significant roles in plant stress responses [1, 56].

The identification of cold-responsive miRNAs came from the pioneer work of sequencing a library constructed from *Arabidopsis* seedlings that had been exposed to cold, dehydration, salinity stresses, and ABA [57]. The expression of miR393, miR397b, and miR402 was upregulated by a number of stress treatments, while miR319c was specifically upregulated by cold. It was also found that miR389a was downregulated by cold and other stresses.

Following this study, a number of laboratories have taken additional approaches such as computational analysis and microarray data analysis to further expand the

number of miRNAs that respond to cold stress treatment. In one study, the authors were able to identify 19 more miRNAs that are potentially cold inducible by intelligently integrating the data from diverse resources, such as microarray data from stress-treated samples, promoter data from cold-responsive protein-coding genes, and bioinformatic predictions of miRNA-target gene pairs (database), and combining them using computational methods such as support vector machine (SVM) and *k*-nearest neighbors (k-NN). Further analyses of the promoters of these 19 miRNAs revealed that more than half of them contain the ABRE and/or CRT/DRE elements, which are known to be cold inducible, confirming the validity of their bioinformatic method [58].

In an independent study, 117 probes corresponding to the same number of *Arabidopsis* miRNAs were spotted on an in-house fabricated cDNA-type microarray. This microarray was then hybridized with small RNAs extracted from samples treated with cold stress [59]. A total of 11 cold-inducible miRNAs were identified from this experiment, and all but two of them were also identified through the integrated computational approach from the first study discussed, suggesting that a skillfully designed computational approach could potentially be employed to accurately and more efficiently annotate the functions of miRNAs in a systematic fashion.

Although these methods have successfully identified a number of miRNAs whose expression is cold inducible, they have barely begun to identify miRNAs whose expression is downregulated by cold stress. Since microRNAs generally act as translational repressors, their targets will represent those genes with expression that is repressed by cold stress. On the contrary, for those target genes playing positive roles during plant cold stress response, their corresponding miRNAs have to be downregulated. These miRNAs certainly should not be overlooked. Microarray technology, as well as the deep sequencing strategy, might be a better way to identify miRNAs that are downregulated during stress [60–62] than traditional approaches such as cloning-based technology or Northern blot analysis [63], illustrated in an example in the next paragraph.

Recent technological advancements have made important contributions to the identification of novel miRNAs in other plant species, especially in those species whose genome sequences were not known. Nevertheless, investigation of these species might reveal novel adaptive strategies plants have evolved in response to environmental stresses. It was proposed that the winter-habit temperate plant *Brachypodium distachyon* might represent a better model monocot system than rice to study plant cold acclimation and cold stress response [61]. A study using high-throughput sequencing with the Solexa next-generation sequencer (www.illumina.com) identified 27 species-conserved miRNAs and 129 species-specific miRNAs from *B. distachyon*. Interestingly, among the conserved miRNAs, three were found to be regulated by cold stress, while the expression of all of them was upregulated. Meanwhile, 25 out of the 129 *Brachypodium* unique miRNAs displayed significant changes following cold stress treatment. However, 76% of them (19/25) had repressed expression following cold stress, suggesting that in *Brachypodium*, mRNA repression is by far the major mode of cold regulation on miRNA expression [61].

5.2.2.4 Posttranslational Regulation

The mRNA expression of transcription factor genes *CBF/DREB1* can be rapidly induced in as short as 15 min following cold treatment. Thus, it is apparent that other mechanisms including posttranslational regulation must also be involved in the regulation of cold-responsive genes. Among all posttranslational pathways, the ubiquitin/proteasome degradation pathway constitutes an essential regulatory mechanism in controlling the activities of central cold-responsive genes such as ICE1 [64]. The involvement of the ubiquitin/proteasome degradation pathway in plant cold response began to be understood when it was discovered that treatment with the proteasomal inhibitor PI II MG132 or DMSO significantly enhanced the promoter activity of the cold-responsive gene RD29A [64]. Later on, it was found that the *Arabidopsis* HOS1 (high expression of osmotically responsive protein 1), which belongs to a family of C3HC4 RING finger domain containing protein, negatively regulates the ICE1 activity through ubiquitination [64]. The *HOS1* gene encodes a functional ubiquitin E3 ligase, which is involved in the last step of ubiquitination. This last step recruits specific target proteins to the ubiquitin complex and directs the ubiquitinated proteins to a proteasome complex for degradation [65]. It was shown through both *in vivo* and *in vitro* studies that HOS1 mediated ubiquitination, and subsequent degradation of ICE1 is functionally involved in the cold signaling pathway [64]. While the *hos1* null mutant displays both enhanced ICE1 protein activity and enhanced cold-responsive *CBF/DREB1* expression, overexpression of the HOS1 gene results in a much more reduced cold response and freezing tolerance. It is also interesting to note that the localization of HOS1 moves from the cytoplasm at warm temperatures to the nucleus after a plant's exposure to cold temperatures, indicating that other regulatory mechanisms, such as protein translocation, are also involved in the HOS1-ICE1 regulatory pathway [64].

Another important gene involved in the ubiquitin-mediated regulation of ICE1 is the *Arabidopsis SIZ1* gene. The *SIZ1* gene encodes SUMO (for small ubiquitin-related modifier) E3 ligase, an enzyme that catalyzes the last step of SUMO conjugation to the protein substrates [66]. The reversible sumoylation/desumoylation of the target proteins determines their activity and final destination and, thus, is involved in many important processes such as transcriptional regulation, protein subcellular compartmentalization, and ubiquitin/proteasome-mediated protein degradation [67]. The *Arabidopsis SIZ1* gene was originally identified as one of the essential genes during plant response to phosphate starvation [68, 69]. Later on, it was found that this gene was also involved in the plant cold stress response since the *siz1* null mutant showed reduced cold and freezing tolerance. In addition, the expression of *CBF/DREB1* genes was also reduced in the *siz1* null mutant. Interestingly, the mRNA expression of a negative regulator, the *MYB15* gene, was substantially higher in the *siz1* mutant than the wild-type plants, suggesting that SIZ1 positively regulates the cold-responsive gene expression by downregulating the expression of *MYB15* gene [66].

Since SIZ1 encodes a SUMO E3 ligase, it was speculated that the SUMO conjugates in the *siz1* mutant might be compromised. As predicted, the antibodies against SUMO1 detected fewer SUMO conjugates in the *siz1* mutant than those in

the wild-type plants, confirming that SIZ1 is indeed involved in the sumoylation process [66]. However, it has not been investigated whether sumoylation is directly involved in the regulation of CBF/DREB1 and MYB15 protein or not. On the other hand, it was found that ICE1 could indeed be sumoylated, and the amount of sumoylated ICE1 is moderately increased by cold treatment. The cold-inducible SUMO conjugation of ICE1 was blocked in the *siz1* mutant if the ICE1 protein was mutated at position 393, which changes lysine (K) to arginine (R) [66].

When the expression of *CBF/DREB1* and *MYB15* was tested, a positive correlation was found between the degree of ICE1 sumoylation and the level of CBF/DREB1 mRNA expression, but there was a negative correlation with MYB15 mRNA expression. The increased sumoylation of ICE1 probably resulted in the enhanced stability of the ICE1 proteins since it was found *in vitro* that polyubiquitination of ICE1, likely mediated by HOS1 or other related proteins, was reduced if ICE1 were sumoylated, as compared to the unsumoylated control. The functional significance of ICE1 sumoylation was further confirmed by reverse genetic studies, where overexpression of the mutant version of ICE1 (K393R) resulted in the reduced freezing tolerance in the transgenic *Arabidopsis* plants [66].

5.3 Signaling Molecules Involved in the Early Events of Cold Stress Response

5.3.1 Phospholipids

A plant cell membrane is comprised of a lipid bilayer containing many integral and peripheral proteins that function as ion channels and membrane receptors. The plant cell membrane is quite fluid and selectively permeable. More than simply being a rigid structure holding the entire cell, the cellular membrane also conveys an abundance of information. This is mainly attributed to one of its predominant lipid components, the phospholipids. Upon exposure to cold stress and other adverse environmental stresses, the production of one of the phospholipids, phosphatidylinositol 4,5-bisphosphate (PIP_2), was found to increase rapidly through the activation of phosphatidylinositol 4-phosphate 5-kinase (PI5K) [70]. The increase in the PIP_2 level subsequently results in the increased production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which is catalyzed by phosphoinositol-specifc phospholipase C (PI-PLC) [71]. It has been shown that both the transcript level and the protein activity of *Arabidopsis* AtPLC1 are coordinately induced by cold stress [71–73]. The DAG and IP_3 are two important secondary messengers that can activate protein kinase C and the release of Ca^{2+} from intracellular storage [74, 75]. The transient surge in the cytoplasmic Ca^{2+} level then triggers a series of signal transduction cascades, which will be discussed next.

In addition to PLC, phospholipase D (PLD) is also involved in the plant stress responses [76]. The phosphatidic acid (PA), generated from phospholipids by PLD, is another important secondary messenger that can activate protein kinase C [77]. In

Arabidopsis plants, it was found that the endogenous PA level correlated well with the expression of one of the *Arabidopsis* PLD genes, *AtPLDδ*. In the *atpldδ* knockout mutant, which had significantly reduced levels of PLD activity, the PA level was also much lower than that of the wild-type plants, and the mutant plants displayed decreased freezing tolerance [78]. On the other hand, transgenic *Arabidopsis* plants overexpressing *AtPLD* have much higher levels of PA than wild-type plants, and also have enhanced freezing tolerance, suggesting that PA and PLD are directly involved in plant cold stress response [78]. Using *Arabidopsis* suspension cells, it was found that the enzymatic activities of both PLC and PLD were rapidly activated following exposure to cold temperature [79]. Interestingly, the PLC- and the PLD-dependent pathways may have different signaling components since treatment with the PLC inhibitor and PLD inhibitor results in the inhibition of the expression of two different sets of genes [80].

The stimulation of PLC and PLD activities can also be achieved through activation of G-protein-coupled receptors (GPCRs) following perception of external stimuli [81]. This will be discussed later in this chapter.

It is also worthwhile to mention that the protein product of the *Arabidopsis Fryγ1* gene, which is directly involved in plant lipid metabolism, also contributes to the cold stress response. The *Fryγ1* gene was identified through a genetic screening for mutants with altered cold stress response [82], and encodes inositol polyphosphate 1-phosphatase (Ins1Pase), an enzyme responsible for the hydrolysis of IP_3. The null *fryγ1* mutant displays an enhanced cold-responsive gene expression, consistent with the notion that IP_3 is an important signaling molecule involved in cold stress response.

5.3.2
Reactive Oxygen Species as Secondary Messenger

Reactive oxygen species (ROS) is generated when free, high-energy electrons are transferred to molecular oxygen (O_2) to form unstable intermediates such as $^1O_2$, H_2O_2, $O_2^{\bullet}-$ and $HO^{\bullet}$. Under optimal plant growth conditions, the level of cellular ROS is kept very low inside the plant cells. However, during exposure to adverse environmental stresses, the ROS level is drastically elevated to an extent that could cause damage to plant cells due to reactions such as cell membrane lipid peroxidation, protein oxidation, and enzyme inhibition [83–85]. As a result, the cellular scavenging system, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase, and glutathione transferase (GST), has to be activated quickly to clear up excess ROS and to restore whole cell redox homeostasis. Many previous reports have shown that by manipulating the mRNA expression levels of genes encoding ROS scavengers, different levels of tolerance to environmental stresses could be observed (reviewed in Ref. [84] and references therein). For example, the *atvtc-1* mutant, which is deficient in cellular ascorbate, is significantly more sensitive to salt stress [86]. On the other hand, transgenic *Arabidopsis* overexpressing the glutathione peroxidase 3 gene (*AtGPX3*) shows increased drought stress tolerance [87].

Interestingly, ROS appears to have a dual function inside the plant cells. In addition to its stress damage to cells, ROS also plays an essential role as a secondary messenger

in stress signaling, and this has already been demonstrated in osmotic, drought, salt, heat, and high light intensity stress studies [84]. One of the examples came from a study of the *Arabidopsis APX1* gene, which codes for a plant ascorbate peroxidase. It was found that, compared to wild-type plants, the *atapx1* knockout mutant accumulated a much higher level of H_2O_2 within the cells, most likely due to a low level of ascorbate peroxidase activity available to scavenge the extracellular H_2O_2. As a consequence, this mutant plant was found to be hypersensitive to light stress [88]. Surprisingly, the *atapx1* mutant appeared to be more tolerant to osmotic, salt, and cold stress, and a number of stress-responsive genes were induced even without stress treatment [89]. This result suggests that higher levels of H_2O_2 might, on the one hand, cause hypersensitivity to oxidative stress but, on the other hand, trigger a ROS-mediated stress signaling pathway that could result in enhanced tolerance to certain types of abiotic stresses.

There have been quite a few studies elucidating the activities of ROS in plant abiotic stress signaling. In animal cells, numerous studies describe ways in which oxidative stress signals, such as H_2O_2, can trigger a wide array of cellular responses mediated by mitogen-activated protein kinases (MAPKs). The plant MAPKs are known to play critical roles in cold stress and other abiotic stress signal transduction. It was found that one of the *Arabidopsis* MAPKs, the AtMPK6, which can be activated by cold or osmotic stress, is also activated by H_2O_2 treatment [90], raising the possibility that ROS, as a secondary messenger, relays abiotic stress signals by acting on downstream signaling molecules such as protein kinases.

Transcription factors also mediate H_2O_2 signaling. In a microarray study in which the authors compared the difference during early responses to chilling stress between chilling-insensitive japonica rice and the chilling-sensitive indica rice, they found that the expression of one of the b-ZIP transcription factors, later called *ROS-bZIP1*, was induced within 2 h of chilling treatment in the japonica ecotype background, but the induced expression was much less robust and delayed in the indica background [91]. Interestingly, the expression of *ROS-bZIP1* was also H_2O_2 inducible. Moreover, the authors also identified a group of genes that responded to chilling stress at a later time point (6 h) and found that the promoters of these genes contained the *as1/ocs*-element, the canonical binding site for b-ZIP-type transcription factors [92], implying that the induced expression of the late-responsive genes might be caused by activating ROS-bZIP through an ROS-mediated pathway.

In addition to the b-ZIP-type transcription factors, the ERF/AP2-type transcriptional factors have also been linked to cellular H_2O_2 responses. The tomato JERF3 protein, which belongs to the ERF/AP2 superfamily of transcription factors, was identified through a yeast one-hybrid screening that bound to the GCC-box of the ethylene response element [93]. This gene, when overexpressed in transgenic tobacco, confers enhanced tolerance to salt, drought, and freezing stresses. Further expression analysis indicated that the enhanced stress tolerance might be a result of the activation of a ROS-mediated pathway since phenomena such as the increased activity of the tobacco SOD and a reduced accumulation of cellular H_2O_2 were observed, as well as the transcriptional activation of ROS-responsive genes [94].

The last category of transcription factors involved in the ROS-mediated signaling pathways during plant abiotic stress responses is that of the C2H2-type zinc finger transcription factors. Two examples of this type are the *Arabidopsis ZAT7* and *ZAT12* genes, which were identified through a microarray data analysis that revealed elevated mRNA expression of both genes in the *atapx1* mutant [95]. Like *AtAPX1*, the mRNA expression of *ZAT7* and *ZAT12* can also be induced upon application of exogenous H_2O_2. In the *zat12* knockout mutant, the inducible expression of *ZAT7* and *AtAPX1* following H_2O_2 treatment was blocked, and the mutant was more sensitive to oxidative stress. In contrast, transgenic plants that overexpress *ZAT12* are more tolerant to oxidative stress, suggesting that ZAT12 is involved in mediating H_2O_2 signaling [95, 96]. Most compelling is the idea that overexpression of the *ZAT12* gene in transgenic *Arabidopsis* plants could result in improvement in freezing stress tolerance, pointing to a central position for ZAT12 in bridging the ROS-mediated cold stress signaling pathway [37].

ZAT7 was shown to possess a capability similar to ZAT12. Overexpression of ZAT7 in transgenic *Arabidopsis* plants also resulted in a dramatic tolerance to salinity and cold stresses [95, 97]. In contrast, ZAT7 RNAi lines showed decreased osmotic stress tolerance [97]. With the support of these data, a comprehensive deletion and mutational analysis of the ZAT7 C2H2-zinc finger domain concluded that this domain was required for abiotic stress response in plants [97].

5.3.3
Calcium Binding Proteins and Signal Decoding

One of the earliest signaling events preceding the onset of transcriptional activation of early cold-responsive genes such as *CBF* expression is a rapid increase in Ca^{2+} concentration in cytoplasm, which happens within the first 5 min after a sudden temperature drop [2, 74, 98]. The transient spike of Ca^{2+} concentration in the cytosol serves as a potential signal that must be perceived by calcium binding proteins, also called calcium sensors, or else the downstream signaling cascades will not occur [99–101].

Typically, the calcium binding proteins contain four signature motifs, called helix–loop–helix EF-hands, which function to physically interact with Ca^{2+} ions. Binding of Ca^{2+} results in conformational changes in the calcium binding proteins for subsequent interaction with other signaling components and relay of the signals. In general, there are three types of Ca^{2+} binding proteins in plants. The first type encompasses the calmodulins and calmodulin-like proteins (CaM and CML, respectively), the second type includes calcium-dependent protein kinases (CDPKs), and the third type is comprised of the calcineurin B-like proteins (CBLs) [102, 103].

CaMs are found universally in both animal and plant kingdoms. They are versatile Ca^{2+} binding proteins involved in numerous cellular processes by binding to effector proteins in response to Ca^{2+} signals [104]. The *Arabidopsis* genome contains 9 CaMs genes and an even larger CML family that contains at least 50 CML proteins. The magnitude of this CML family suggests that they are probably required for many cellular and physiological responses; however, the functions for many of these CMLs are not known [105, 106].

CML24 appears to be involved in a number of stress responses [107]. This gene was first identified as a touch-inducible gene and later its mRNA expression was found to be induced by other stresses, including cold and heat. Transgenic plants over- or underexpressing the *CML24* gene display a variety of phenotypes. However, its direct involvement in cold stress response has not been tested. In addition to CML24, the *Arabidopsis* CML9 gene was also shown to be involved in plant abiotic stress responses and response to treatment with plant hormone ABA [108].

The second group of the Ca^{2+} binding proteins contains the calcium-dependent protein kinases. CDPKs are serine/threonine protein kinases characterized by phosphorylation activities that are altered with Ca^{2+} binding. Previous studies have shown that a number of plant CDPKs are activated following cold stress treatment [109–111]. A piece of direct evidence came from a study in which overexpression of the rice *OsCDPK7* gene resulted in the induced expression of a number of stress target genes, which correlated with enhanced cold, drought, and salt tolerance in the transgenic rice plants [112, 113]. However, questions still remain: what are the downstream phosphorylation targets for CDPKs and how are CDPK proteins connected to other signal transduction molecules such as CBF/DREB1 transcription factors? A search for proteins that interact with CDPKs was initiated using a yeast two-hybrid system and did yield some clues as to how the stress signals were relayed from CDPK protein kinases. One study determined that *Arabidopsis* CDPK32 interacts with AREB4/ABF4, a bZIP transcription factor involved in the ABA response [114]. CDPK32 phosphorylated AREB4/ABF4 *in vitro* on the serine residue at position 110. Phosphorylation at this particular site was functionally important since a mutation at Ser110 dramatically reduced ABF4 transcriptional activity in protoplasts [114]. Using a slightly modified yeast two-hybrid approach, a zinc finger nuclear protein, AtDi19, was identified that interacts specifically with the *Arabidopsis* CDPK4 and CDPK11 proteins [115]. In previous studies, the expression of AtDi19 was shown to be drought inducible, but the inducible expression was independent of ABA [115]. Thus, it appears that some plant CDPKs can also function through an ABA-independent stress pathway [116]. A recent study was performed that systematically identified potential target proteins that were differentially phosphorylated by CDPKs when the plants were subjected to salt treatments [117]. The authors compared the whole-cell threonine phosphorylation pattern of wild-type *Arabidopsis* to that of *atcpk3* mutants and found 28 proteins that were possibly phosphorylated by AtCDPK3 following the salt stress treatment [117]. Interestingly, most of these proteins were predicted to be membrane associated; however, the annotation of these 28 proteins did not yield much valuable information in regard to how CDPKs might function in any known cold response pathway [117].

5.3.3.1 The Calcineurin B-Like Proteins in Decoding the Ca^{2+} Signals

Finally, the last group of Ca^{2+} binding proteins is that of the CBLs. Unlike CDPKs, these proteins do not possess protein kinase activities [103]. Many studies have demonstrated that CBL proteins, which are unique to plants, are involved in plant responses to multiple abiotic stresses, including cold, salt, drought, and osmotic stresses [118–122]. *Arabidopsis* CBL1 is one of the best-studied CBL class of proteins

pertaining to plant abiotic stresses. The *CBL1* gene was first identified through a homology screen using primers designed from the conserved regions of calcineurin B genes of animal species [121]. *CBL1* contains four typical EF-hand-like sequences and functionally binds to Ca^{2+}. The mRNA and protein expression of the *CBL1* gene is induced by cold, drought, and wounding [120, 121]. The involvement of CBL1 in plant stress responses was further confirmed by the characterization of both *cbl1* mutant plants and *CBL1*-overexpressing transgenic plants [118, 120]. Notably, the CBL1 protein appears to differentially regulate plant responses to different abiotic stresses. Although CBL1 functions as a positive regulator in salt and drought stress responses, its role in cold stress response appears to be negative since the overexpression of *CBL1* results in a reduced tolerance to freezing. By contrast, the *cbl1* null mutant displays enhanced freezing tolerance, in concert with enhanced *CBF*/*DREB1* gene expression [118, 120].

What interacts with CBL proteins and how are stress signals relayed to the downstream components in the signaling pathway? The search for CBL interacting proteins with conventional methods, such as yeast two-hybrid screening, yielded rapid results in the identification of the CBL-interacting protein kinases (CIPKs) in *Arabidopsis* and other plant species [123]. The CIPKs belong to the class of serine/threonine protein kinases, which contain an N-terminal kinase catalytic domain and a C-terminal regulatory domain. It is the C-terminal regulatory domain that is responsible for the interaction between CBLs and CIPKs, and such interaction depends on Ca^{2+} [119, 124, 125]. Indeed, CIPKs are the primary target kinases of CBL proteins upon exposure to high concentrations of Ca^{2+} ions [126]. For most CIPKs, the downstream substrates are not clear, nor how they relay the Ca^{2+} signals. The *Arabidopsis* SOS1 protein is among the very few CIPK targets identified so far (discussed below).

Another intriguing observation from the study of the *Arabidopsis* and the rice genomes is that it appears that the plant genome contains large CBL and CIPK families. In *Arabidopsis*, there are at least 10 members of the CBL family and at least 25 members in the CIPK family [127]. The rice genome contains a similar number of CBL proteins, but the size of CIPK family is bigger than *Arabidopsis* (30 proteins) [127].

So how is the specificity of cellular responses determined when the concentration of Ca^{2+}, a universal signal molecule, increases upon multiple stress stimuli, such as cold, salt, drought, and wounding? In other words, how are the different Ca^{2+} signals decoded? Specific interactions between CBLs and CIPKs might account for part of the specificity [124, 126]. It was found that the two highly similar CBLs, CBL1 and CBL9, interact exclusively with both CIPK1 and CIPK23; that is, they do not interact with any other CIPKs [122, 128]. While the interaction between CBL1/CBL9 and CIPK1 is involved in osmotic stress response [128], the CBL1/CBL9-CIPK23 complex is involved in the regulation of plant potassium (K^+) channel and water loss in guard cells and root hairs [129–132]. CBL10 interacts only with CIPK24 during salt stress, and such interaction subsequently results in sequestration/compartmentalization of Na($^+$) in plant vacuoles [133]. However, CIPK24 also interacts with CBL4, also known as SOS3 [134–137]. The *Arabidopsis CIPK24*, also known as *SOS2*, was identified

through the same genetic screen where the P_{RD29A}-LUC system was applied [138]. It was found that the catalytic and regulatory domains of the CIPK24/SOS2 protein interact with each other under nonstress conditions, probably by blocking the kinase activity of CIPK24/SOS2 [139, 140]. The interaction between CIPK24/SOS2 and CBL4/SOS3 upon an internal Ca^{2+} spike relieves the repression and activates CIPK24/SOS2 kinase activity [139]. The activated CIPK24/SOS2 then phosphorylates the SOS1 protein, a Na^+/H^+ antiporter, which finally leads to salt tolerance [141, 142]. The CBL4/SOS3- CIPK24/SOS2-SOS1 represents one of the best-studied salt stress pathways in plants [25, 101, 143].

Another hypothesis for specificity is based on distinct target sequences located at the N-terminal domain of the CBL proteins. While some of the CBL proteins contain N-terminal myristoylation and S-acylation signals that target them to the plasma membrane, some CBL proteins do not, suggesting that their corresponding CBLs/CIPKs might function in the cytoplasm [119, 144]. For example, CBL1, CBL4/SOS3, CBL5, and CBL9 are all located on the plasma membrane, whereas CBL2, CBL3, CBL6, and CBL10 are located on the vacuole membrane. The last two CBLs, CBL7, and CBL8 are located in the cytoplasm [144]. The discrete localization of the CBLs/CIPKs complexes might be an important strategy plants adopt to decode Ca^{2+} signals in response to specific developmental and environmental stimuli. This is exemplified in the interaction between CBL4/SOS3, CBL10, and CIPK24/SOS2. CIPK24/SOS2 can interact with both CBL10 and CBL4/SOS3, but its final cellular localization is different. Upon salt stress, in the root cells, CBL4/SOS3 directs CIPK24/SOS2 to the plasma membrane where CIPK24/SOS2 exerts its function by phosphorylating the SOS1 Na^+/H^+ antiporter [2, 141, 143]. However, in shoots, upon the same salt stress, CBL10 directs CIPK24/SOS2 not to the plasma membrane but to the vacuolar membrane (tonoplast), in order to control intracellular salt homeostasis [133].

5.3.4 Mitogen-Activated Protein Kinase Proteins: Essential Roles in Plant Stress Responses

The MAPK proteins have received a substantial amount of attention in regard to plant stress responses, and this is mainly due to the critical roles their counterparts play in a variety of yeast and animal stress signal transduction pathways and networks [145, 146]. The MAPK pathway is typically comprised of three linearly organized components. The last component in the pathway, the MAPK, is activated by phosphorylation at threonine and tyrosine residues in the conserved T-x-Y motif, by the middle component, the MAPK kinase (MKK). The MAPK kinase/MKK in turn is regulated by phosphorylation on the two serine/threonine residues by the upstream MAPK kinase kinase (MAPKKK or MEK).

A variety of approaches were employed to investigate the possible involvement of MAPKs in plant stress responses. Using yeast two-hybrid analysis and functional complementation studies, as well as protoplast transient expression systems, it has been shown that *Arabidopsis* contains complete sets of functional MAPK cascades that may play roles integral to various biotic and abiotic stress signal transductions [147–150].

To date, two complete sets of MAPK cascades have been characterized in *Arabidopsis*. The AtMEKK1–AtMKK4/AMKK5–AtMPK3/AtMPK6 cascade has been shown to be specifically involved in plant response to bacterial flagellin elicitor in a protoplast system [151], while the AtMEKK1–AMKK2–AtMPK4/AtMPK6 has been shown to be preferentially activated by abiotic stresses, such as cold or salt, in a transient assay combined with mutant analysis [152]. Notwithstanding this result, how AtMEKK1 interprets different stress signals and activates corresponding downstream MAPK pathways still remains to be answered. The mRNA expression for most of the components in MAPK cascades does not change in response to stress treatments, except for AtMEKK1, which is strongly induced by cold [153, 154]. Protein levels for most of the components also stay constant; however, their kinase activities were found to be rapidly turned on following different stress treatments, strongly suggesting that posttranslational modification is mostly responsible for the change. The kinetics for most of the kinase activation is consistent with what was hypothesized previously: that *de novo* protein synthesis is not required for the activation of key transcription factors such as *CBF* genes. These results also place the MAPK cascades as a potential upstream event required for the activation of transcription factors such as *ICE1* and *CBFs*.

One of the more controversial MAPKs in stress responses is AtMPK4. Analysis of the *mpk4* null mutant revealed a negative role of this gene in plant response both to pathogen infection [155] and to hyperosmotic stress [156]. However, the positive role of AtMPK4 in cold and salt stresses was also suggested by another study where it was shown that AtMPK4 was one of the direct and preferred phosphorylation targets by the MAPK kinase, AtMKK2 [152]. AtMPK4 also strongly interacted with AtMKK2 in a yeast two-hybrid system. Constitutive activation of AtMKK2 resulted in the strong activation of AtMPK4 kinase activity, as well as the induced mRNA expression of a number of cold stress marker genes such as *CBF2* and *CBF3* [152]. These results implied that AtMPK4 might play very specific roles in response to various types of stresses. Similar findings have been reported with the rice *OsMAPK5* gene, where it was shown that disease resistance and abiotic stress tolerance appear to be inversely modulated by the rice OsMAPK5 [157]. Nevertheless, the role of AtMPK4 in directly mediating AtMKK2 activity in the expression of stress-responsive genes requires further examination.

Regarding the direct targets or phosphorylation substrates downstream of the MAPK cascades, only limited studies have been reported so far. Through a yeast two-hybrid screening, an AtMPK4 interacting protein, named MKS1, was identified, which might mediate the expression of some WRKY-type transcription factors in plant defense response [158]. In addition, it was shown that AtMPK4 could phosphorylate MKS1 *in vitro*, suggesting that MKS1 acts downstream of AtMPK4. However, the specific role of MKS1 phosphorylation by AtMPK4 in defense signal relay was not investigated. Systematic approaches such as protein microarray-based methods have also been developed to identify target substrates of MAPKs. Using this approach, 48 potential substrates of AtMPK3 and 39 of AtMPK6 were identified [159]. Although the aim of this study was to identify proteins involved in the inflorescence meristem development mediated by AtMPK3 and AtMPK6, a similar approach can nevertheless be applied to identify potential MAPK targets.

5.3.5
Do Ca^{2+}-Mediated Signaling Pathways Crosstalk with the MAPK-Mediated Pathways?

The CDPK-mediated stress signaling and MAPK-mediated signaling represent two major signaling pathways activated in many organisms in response to stress conditions. In animals, voluminous data clearly showed that there are crosstalks between these two pathways [160]. However, it does not seem to be the case in plants, especially during plant response to salt stress [117]. It was found that the *atcdpk3* mutant displayed a salt-sensitive phenotype similar to the *atmkk2* mutant. However, mRNA expression of a number of genes that were known to be regulated at transcriptional levels by *MKK2* remained unchanged in the *atcdpk3* mutant, indicating that *AtCDPK3* is not involved in the same mode of transcriptional regulation of the salt-responsive genes. The subsequent proteomic analysis identified 28 proteins with altered phosphorylation patterns between wild-type and *atcdpk3* mutant plants. Interestingly, most of these proteins were predicted to be membrane located, for example, VDAC1 (voltage-dependent anion channel 1) and VDAC2 [117]. At the same time, it was found that the AtCDPK3 protein itself contains a myristoylation site and a substantial amount of this protein was membrane located [117]. This prompted the authors to propose the hypothesis that while the MAPK pathway is primarily responsible in mediating transcriptional regulation of salt-responsive genes during salt stress response, CDPKs might regulate mainly through the direct activation of membrane-associated proteins such as ion channels [117]. However, it is not clear if this hypothesis also works for plant responses to cold, drought, or osmotic stresses.

5.4
Other Signaling Molecules Involved in Cold Signaling Pathways

A large body of evidence strongly suggests that cold and other abiotic stress signaling pathways are extremely complicated, and legions of signaling molecules are involved in transducing specific external signals to their corresponding targets, in order to trigger the right cellular responses. Thus, beyond the essential signaling molecules that have been discussed in the previous sections, I will also mention a few additional members that most likely play pivotal roles in plant cold and abiotic stress responses.

5.4.1
MAPK-Specific Phosphatases and Other Protein Phosphatases

As already discussed, MAPKs are involved in the signaling of many physiological processes, including plant response to abiotic stress. However, continuous activation of MAPK pathways under unnecessary conditions is costly and detrimental to plants; thus, the activities of MAPKs must be tightly regulated. While MAPKs are activated by MKKs through phosphorylation at the threonine and tyrosine residues, they are inactivated by the MAPK-specific phosphatases (MKPs). MKPs belong to a group of

dual-specificity phosphatases (DSPs; Ser/Thr and Tyr), and they dephosphorylate MAPKs at the same amino acid residues [161]. There are 22 DSPs in the *Arabidopsis* genome; however, only 5 of them were functionally characterized [161]. Of these, MKP1 is most likely involved in plant response to abiotic stress [162]. MKP1 is able to dephosphorylate MAPK6 both *in vitro* and *in vivo*, and an *mkp1* mutant displays enhanced resistance to salt stress, consistent with the hypothesis that MAPK6 is a positive regulator in plant abiotic stress response and that the regulation depends on its kinase activity. An increase in MAPK6 activity in the *mkp1* mutant background would then be expected to lead to increased abiotic stress tolerance. Microarray analysis confirmed this idea and showed that the expression of a number of salt stress-regulated genes was induced in the *mkp1* mutant background [162].

In addition to the MKPs, other protein phosphatases are involved in the inactivation of MAPKs, thus controlling the balance between active and inactive MAPKs. The PP2C plant family of serine/threonine protein phosphatases constitutes another group of phosphatases that can dephosphorylate threonine residues within the T-x-Y motif of MAPKs. The *Arabidopsis* PP2C-type phosphatase AP2C1 can inactivate MAPK4 and MAPK6 *in vitro* and *in vivo*, and increased levels of AP2C1 result in a compromised response to pathogen infection in the *AP2C1* overexpression transgenic *Arabidopsis* plants [163]. It is not clear if AP2C1 is involved in plant responses to abiotic stresses. However, since both MAPK4 and MAPK6 are involved in abiotic stress signaling, and AP2C1 negatively regulates MAPK4 and MAPK6 activities, it is likely that AP2C1 might also be involved in plant abiotic stress responses.

Another group of PP2C-type phosphatases, the plant tyrosine-specific phosphatases (PTPs), were also shown to be able to deactivate MAP kinase activities. One example came from the study of the *Arabidopsis* PTP1 protein. Interestingly, the mRNA expression level of *AtPTP1* is upregulated by salt stress but downregulated by cold stress [164]. The AtPTP1 protein was also found to interact with MAPK6 in a yeast two-hybrid study, suggesting that MAPK6 might be the target of AtPTP1 to regulate plant abiotic stress responses [165].

5.4.2
Two-Component Systems

The two-component sensor regulator system was originally found in prokaryotes, and this predominant signal transducing system was found to be involved in response to different environmental stimuli [166]. However, a large body of experimental data has demonstrated that eukaryotes, including plants and animals, also use two-component systems (TCS) to transduce external signals [167–169]. The conventional TCS found in most of the prokaryotic organisms is typically composed of two proteins, a membrane-localized histidine kinase (HK), which senses the input signals, and an aspartate response regulator (Asp-RR), which is normally a transcription factor that outputs the signal. The perception of external signals by HK causes an autophosphorylation of its own His residue, effecting transfer of the phosphoryl group to the Asp residue, which then leads to the activation of the Asp-RR. However, in most of the eukaryotes, the two-component system has evolved to include an intermediate

component that sits between HK and Asp-RR. This protein is an independent histidine phosphotransfer (HPT) protein [168].

In *Arabidopsis*, proteins homologous to all the components of the eukaryotic two-component system have been identified [167]. One of the most thoroughly studied functional two-component systems in plants is the cytokinin signaling pathway, where the plant phytohormone cytokinin signal is perceived by a HK and transduced to an Asp-RR through an intermediate HPT [170, 171].

Multiple pieces of evidence point to the plant two-component system as being involved in abiotic stress responses, yet a complete and functional HK-HPT-Asp-RR is still lacking [172–174]. For example, the *Arabidopsis* HK protein, AtHK1, which is essential for cytokinin signal perception, is also involved in drought, osmotic, and salt stress responses, both in an ABA-dependent and in an ABA-independent manner [175]. This is further supported by microarray data analysis demonstrating that the mRNA expression of many abiotic stress- and/or ABA-inducible genes, including *AREB1*, *ANAC*, and *DREB2A* transcription factors genes, is downregulated in the *athk1* mutant [175]. Further expression analysis of the *Arabidopsis HK2*, *HK3*, and *HK4* genes in response to various abiotic stresses showed that the transcripts of all three *AtHK2*, *AtHK3*, and *AtHK4* were rapidly induced by dehydration [173]. Expression of *AtHK2* appears to be regulated by salt stress and ABA treatments, and *AtHK3* mRNA is induced by salt and cold stress treatments [173]. What is not clear is that if the HPTs and Asp-RRs work together with the AtHK1-4 during stress responses.

Not much data have been presented so far from the investigation of Asp-RR protein involvement in plant abiotic stress responses. The rice *OsRR6* gene is among the few plant response regulator genes being studied, which may participate in abiotic stress signaling [176]. The mRNA expression of this gene is induced upon cold, salt, and drought stress treatments [176]. The *Arabidopsis* calmodulin-like protein, AtCML9, was previously shown to be involved in abiotic stress response [108]. Using a yeast two-hybrid system, an AtCML9-interacting protein was identified [177]. The corresponding gene encodes AtPRR2, a pseudo response regulator [177]. TheAtPRR2 sequence resembles an authentic plant response regulator; however, it lacks the conserved Asp residue that serves to accept the phosphoryl group [177]. The functional significance of the interaction between CML9 and PRR2 has not been investigated.

An interesting story about the plant Asp-RRs springs from a study between salt-sensitive and salt-resistant ecotype rice [178]. The authors discovered that the expression patterns for all putative members of the rice two-component system, including genes encoding HKs, HTPs, and Asp-RRs, changed in response to stress treatments. More significantly, the mRNA expression of nearly all the two-component system genes is higher in the salt-resistant ecotype rice than in the salt-sensitive ecotype rice, suggesting that these genes might play a role in rice salt stress response.

5.4.3
Heterotrimeric G-Protein-Mediated Signaling in Plant Abiotic Stress Responses

The heterotrimeric G-protein complex is composed of three different subunits: G-alpha (Gα), G-beta (Gβ), and G-gamma (Gγ). It is somewhat surprising that unlike

animal systems, which include multiple members of each subunit gene family (the alpha gene family has 20 members [180], the beta has 5 [180], and the gamma has 12 [181]), the plant genome contains only one or, at most, two members in each subunit gene family. For example, the *Arabidopsis* genome contains only one canonical Gα gene, the *AtGPA1* [182], one Gβ gene, the *AGB1* [183], and two Gγ genes, the *AGG1* and *AGG2* [184, 185]. However, it appears that the plant heterotrimeric G-proteins are involved in a wide range of developmental processes, such as ABA signaling and cell cycle regulation [186, 187].

Recently, their roles in plant responses to environmental signals have begun to emerge [188, 189]. For example, it was shown that the mRNA expression of the pea Gα and Gβ genes was upregulated by heat and salt stresses, as well as by H_2O_2 [189]. Moreover, the overexpression of the pea Gα and Gβ genes in transgenic tobacco resulted in enhanced salt stress tolerance [189], clearly demonstrating that G-proteins are directly involved in plant abiotic stress responses. A Gα gene cloned from *B. napus* [190] also displays interesting expression patterns. Although drought and salt stresses induce the Gα mRNA expression, heat and cold stresses inhibit the expression, suggesting that the functions of G-proteins are specific to various environmental signals [190].

How are the activities of G-proteins regulated? In animal cells, the three G-protein subunits form a complex under regular conditions, with GDP bound to the Gα subunit. In this state, the G-protein is inactive. Once the external stimuli are sensed by a group of proteins called G-protein-coupled receptors, the GPCRs in turn activate Gα subunits by exchanging their bound GDPs for GTPs. The activated Gα subunit then dissociates itself from the Gα–GβGγ complex and subsequently activates its downstream effectors [191, 192].

In mammalian systems, GPCRs play extremely important roles in a multitude of signal transduction pathways. There are about 1000 GPCR genes in human genome [193]. However, there is only a single GPCR gene, called GCR1, to be found in *Arabidopsis* [194], regardless of the existence of 394 divergent GPCR candidate genes [195]. A typical GPCR protein contains seven transmembrane domains, which function in ligand binding, followed by a cytoplasmic domain that is involved in the Gα protein subunit interaction. The binding of ligands to the GPCR proteins initiates their activation, which in turn activates the Gα subunits, and consequently triggers a cascade of cellular responses [81, 193].

Some exciting results were produced in Assmann's laboratory, where they discovered that two *Arabidopsis* GPCR proteins, GTG1 and GTG2, are ABA receptors, which bind ABA specifically *in vitro* [196]. In addition, these proteins also interact with the only *Arabidopsis* G-protein, AtGPA1, suggesting that the ABA signaling might be initiated with the membrane-located GPCR proteins, GTG1 and GTG2, and then relayed to AtGPA1 [196].

So how do G-proteins communicate specific abiotic stress signals? It was found that the alpha subunit of G-proteins could physically interact with phospholipase C (PLC) in both an *in vitro* assay and an *in vivo* assay [189], suggesting that G-proteins relay external signals by stimulating the activities of PLCs. As already discussed in previous sections, the activation of PLCs results in the production of two important

secondary messengers, DAG and IPs, both of which are involved in the release of intracellular Ca^{2+} storage into the cytosol. The sudden increase in cytoplasmic Ca^{2+} triggers a series of signaling cascades, which then leads to final physiological changes inside the cells [81].

PLD also interacts with G-proteins [197, 198]. An *Arabidopsis* PLD, AtPLDα1, preferentially interacts with the inactive GDP-bound Gα subunit, whereas the GTP-bound Gα subunit inhibits the PLDα1 activity [198]. The physical interaction between GDP-Gα and PLDα1 was found to be functionally involved in the ABA-mediated stomatal opening of guard cells [199]. However, direct evidence of any functional interactions between G-proteins and PLCs/PLDs in plant abiotic stress responses is still lacking.

5.4.4 Receptor-Like Protein Kinases

Receptor-like protein kinases (RLKs) constitute one of the largest protein families in plants. There are predicted to be about 600 RLK encoding genes in the *Arabidopsis* genome and more than 1100 genes in the rice genome (see Ref. [200] and references therein). Owing to the presence of a large number of RLKs in plant genomes, it is believed that they are the major components of signaling perception and transduction. As being the predominant class of membrane receptors, RLKs feature an extracellular domain, a single transmembrane domain, and a cytosolic Ser/Thr protein kinase domain.

The plant RLKs possess highly diverse functions in growth and development, and biotic stress responses, as well as in nodulation and rhizobial symbiosis [201]. Their involvement in abiotic stress responses has also been widely documented in literature. In one example, the *Arabidopsis RPK1* gene was rapidly induced upon ABA, dehydration, salt, and cold stress treatments [202]. In another example, overexpression of *RPK1* in transgenic *Arabidopsis* conferred increased ABA sensitivity, drought tolerance, and increased tolerance to oxidative stress, while the knockout *rpk1* mutant displayed opposite phenotypes [203]. Apparently, the RPK1 effect on drought tolerance depends on its kinase activity since a mutation on the conserved lysine residue within the kinase domain abolished this effect [203].

Recently, a detailed gene expression profiling study was conducted by spotting 604 *Arabidopsis RLK* genes on a Syngenta custom GeneChip® microarray, to understand, on a global scale, the transcriptional regulation of the entire complement of *Arabidopsis RLK* genes simultaneously in response to a wide range of environmental and developmental stimuli [204]. The mRNA expression of a significant portion of *RLKs* was found to be regulated during abiotic stress treatments. What is intriguing is that a majority of the *RLKs* responded to more than one signal treatment [204]. Salt stress and cold stress had more shared responses, disclosing 6 (10%) RLK genes with increased expression levels and 27 (19%) with decreased levels. Cold and osmotic stress revealed 3 shared respondents (5%) with increased expression and 16 (8%) with decreased expression. When all the stress treatments were grouped together – biotic, abiotic, and hormonal – it was found that 48 RLKs were induced, while 83 genes were

repressed, by both biotic and abiotic stress and hormone treatments [204], suggesting that most of the *Arabidopsis* RLKs likely perform multiple functions inside the cells.

5.5 Conclusions and Prospects

Abiotic stresses, such as cold, high salinity, drought, osmotic, and heat stresses pose tremendous challenges to plants in their daily lives, negatively affecting their growth, development, and reproductivity. Plants have evolved a battery of mechanisms to adapt themselves to such adverse environments. Through genetics, molecular, biochemical, physiological, and functional genomics approaches, a large number of genes and molecules were discovered that carry out diverse sets of functions during plant responses to abiotic stresses. These molecules, including transcription factors, protein kinases, ubiquitination/proteolysis-related proteins, osmolytes, and other cellular protectants, and even small RNAs, function at various stages of stress signal transduction and responses (Figure 5.1). A thorough understanding of molecular and biochemical mechanisms of plant cold and other abiotic stress responses will be a prerequisite for efficiently designing strategies for improving plant abiotic stress tolerance. However, the number of molecules that have been identified is believed to be far below than what plant scientists expected.

The forward genetics approach, that is, mutant screening for phenotypes, has proved extremely useful in identifying gene components during stress signal transduction. The P_{RD29A}-LUC system discussed in this chapter demonstrates the power of genetic screening in uncovering genes and proteins that play various roles during plant abiotic stress responses. Unfortunately, this approach might have limitations in studying plants, due to problems such as gene functional redundancy, a consequence of the existence of multiple gene families with a large number of members. Reverse genetic approaches, that is, transgenic plants overexpressing a particular gene, or RNAi to silence a particular gene, have been widely used to complement the forward genetic approach to study gene functions. However, previous reverse genetic studies have been limited to individual genes of interest, and systematic analysis of functions of genes that belong to large gene families, such as the CBL family and the RLK family, is still lacking. A number of international consortiums have been organized to focus on tackling some of these large gene families in *Arabidopsis*, and data released from these consortiums to the public database in the future will be extremely beneficial to the plant research community.

Protein kinases are indispensible to plant abiotic responses. Although significant progress has been made toward understanding the mechanisms protein kinases employ during stress signal transduction, many questions remain. A central question is what is the connection between protein phosphorylation and transcriptional activation of stress-responsive genes? Clearly, a major priority is the search and identification of proteins and substrates that are specifically phosphorylated by protein kinases following stress treatment. This information is greatly needed in producing a more complete picture of cold and other abiotic signal transduction

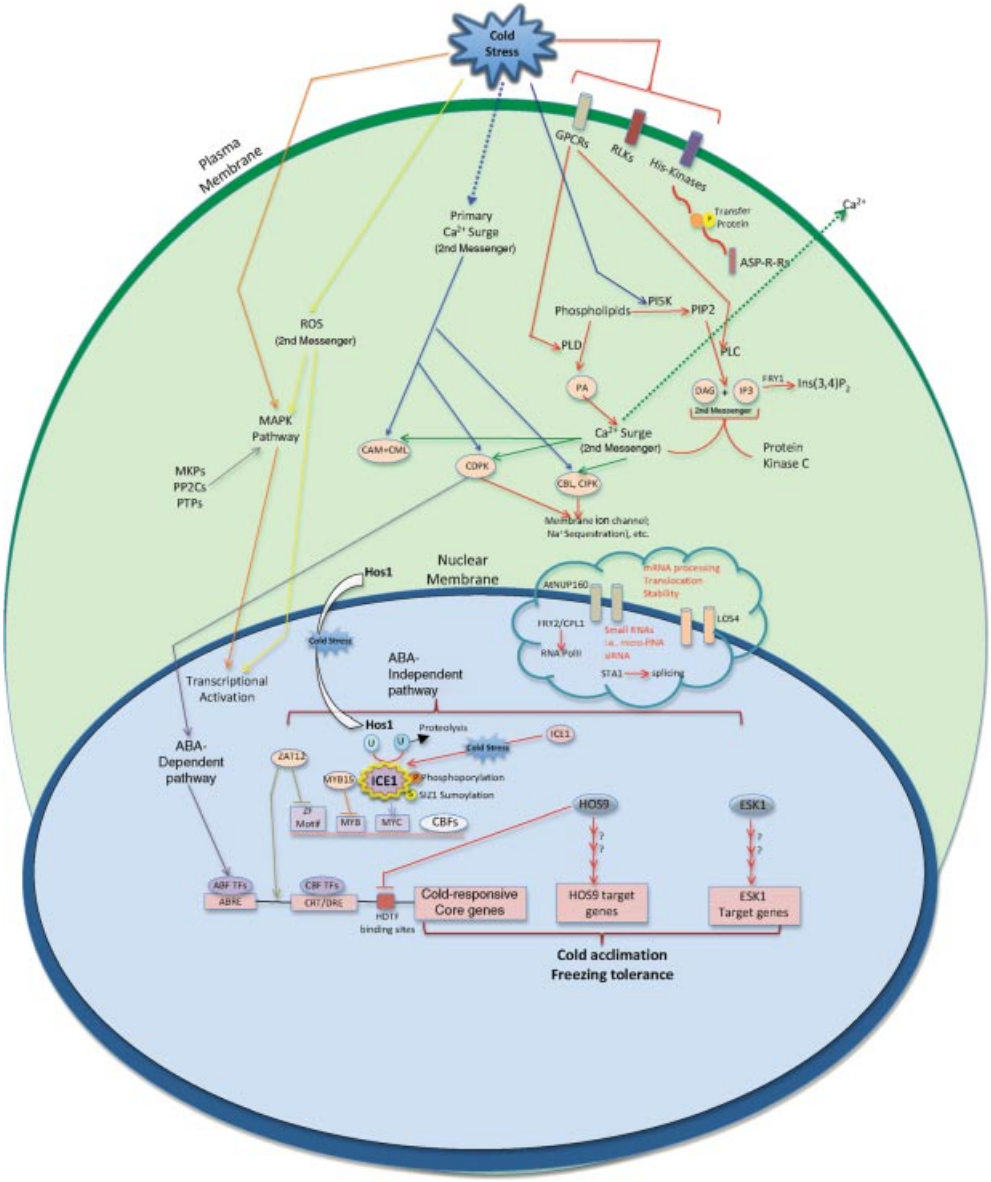

Figure 5.1 Diagram of plant cold stress signaling pathways. Plants might sense cold stress signal through membrane-located receptors, such as GPCRs, RLKs, and histidine kinases (HKs) of the two-component systems. GPCRs regulate PLC and PLD activities through direct protein–protein interaction. Cold stress directly stimulates PI5K activity. PI5K catalyzes phospholipids into PIP2, which in turn is metabolized to two secondary messengers, DAG and IP_3 by PLCs. Cold stress also stimulates PLD activities to produce another secondary messenger, phosphatidic acid. Intracellular Ca^{2+} surge occurs rapidly following cold stress. This Ca^{2+} surge is achieved one way by pumping Ca^{2+} from

networks. Approaches such as the yeast two-hybrid screening have been employed to identify kinase substrates. One of the drawbacks, however, is that often the screening results gained in the identification of proteins have nothing to do with protein phosphorylation. Methods aimed toward specifically identifying phosphoproteins are sorely needed.

Recent technological advancements in the proteomics field, such as the development of a number of methods for phosphopeptide enrichment (including immobilized metal–ion affinity chromatography (IMAC) and TiO_2 chemo-affinity chromatography) coupled with mass spectrometry, made possible the analysis of entire phosphoproteomes. One of the first studies in *Arabidopsis* by Nühse *et al.* (2004) [205] provides an excellent example of applying the IMAC method and mass spectrometry in the identification of phosphoproteins from *Arabidopsis* plasma membrane following fungal elicitor treatment in a cell suspension system. Since many of the receptor-like protein kinases were expected from the elicitor-triggered signaling response, the identification of a large number of phosphorylated RLKs validated this as a powerful approach in phosphoproteomic research [205]. Although future work is still needed to investigate more complex samples from whole cell extracts, and to make quantitative comparisons of the amount of phosphoproteins under different conditions, the results of this work demonstrate that this approach could be a promising alternative to many conventional methods in yielding new insights into the regulatory mechanisms of phosphorylation in stress signaling transduction.

So far, majority of the research on plant abiotic stress responses focuses on *Arabidopsis*, a model plant but economically unimportant. Plants such as soybean,

periplasma space into cytosol. In addition, DAG and IP_3 stimulate Ca^{2+} release from intracellular storage. Cytosolic Ca^{2+} serves as another secondary messenger to activate calcium binding proteins, including CaMs and CMLs, calcium-dependent protein kinases (CDPKs), and CBLs. CBLs and their CIPKs act on membrane ion channels to keep the cellular ion homeostasis. CDPKs, on the one hand, may directly act on membrane ion channels. On the other hand, they may interact with transcription factors to activate stress gene expression, in an ABA-dependent manner. Cold stress also increases the levels of ROS, which subsequently activate stress gene expression at transcriptional levels. Mitogen-activated protein kinase pathways are activated by cold stress too. MAPK pathways might be directly involved in transcriptional activation of cold stress gene expression.

Inside nucleus, the constitutively expressed ICE1 protein is activated by cold stress through phosphorylation and sumoylation. SIZ1 is responsible for sumoylating ICE1. Proteins that phosphorylate ICE1 remain to be identified. ICE1 is a positive regulator of CBF gene expression, while MYB15 and ZAT12 are negative regulators. However, ZAT12 positively regulates the expression of some CORE cold stress-responsive genes. HOS1 translocates from cytosol to nucleus upon cold stress and then inactivates ICE1 through ubiquitination-mediated proteolysis. HOS9 and ESK1 function through ICE1/CBF-independent pathways and positively regulate plant responses to cold stress by activating genes that are different from CBF regulons. HOS9 also counteracts on some of the genes within the CBF regulons. The ICE1/CBF pathway, HOS9-, and ESK1-mediated pathways are independent of abscisic acid (ABA). The ABA-dependent pathway is also responsible for activating cold stress gene expression, and this pathway functions through ABRE binding factors (ABF transcription factors or ABF TFs).

corn, rice, and tomato received less attention due to technological difficulties and less functional genomics resources compared to *Arabidopsis*. Although plant scientists hope to extend the *Arabidopsis* work to these economically important plants, apparently there are differences between *Arabidopsis* and other plant species. For example, the *Arabidopsis AtCBF3* gene was found to be the homologue of the tomato *LeCBF1-3*. However, while overexpression of the *LeCBF1* gene in transgenic *Arabidopsis* results in the enhanced freezing tolerance, overexpression of either the tomato *LeCBF1* gene or the *Arabidopsis AtCBF3* gene in transgenic tomato did not result in the expected enhanced stress tolerance [18].

International efforts for functional genomics studies on genes from economically important plant species are urgently needed since these plant species are directly related to human lives. Hundreds and millions of dollars are lost each year as a result of yield loss due to adverse abiotic stress challenges. Meanwhile, the world population is increasing rapidly, and it is possible that we might be facing food shortages in the near future. Thus, a complete understanding of the molecular and genetic basis of abiotic stress responses in economically important plant species is crucial to the development of genetically modified plants as a major solution to successfully overcome such alarming situation.

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References

1 Chinnusamy, V., Zhu, J., and Zhu, J.K. (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci.*, **12** (10), 444–451.
2 Xiong, L., Schumaker, K.S., and Zhu, J.K. (2002) Cell signaling during cold, drought, and salt stress. *Plant Cell* (14 Suppl), S165–S183.
3 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.*, **57**, 781–803.
4 Chen, W. *et al.* (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell*, **14** (3), 559–574.
5 Fowler, S. and Thomashow, M.F. (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell*, **14** (8), 1675–1690.
6 Hannah, M.A. *et al.* (2006) Natural genetic variation of freezing tolerance in *Arabidopsis*. *Plant Physiol.*, **142** (1), 98–112.
7 Kreps, J.A. *et al.* (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol.*, **130** (4), 2129–2141.
8 Seki, M. *et al.* (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length

cDNA microarray. *Plant J.*, **31** (3), 279–292.

9 Hannah, M.A., Heyer, A.G., and Hincha, D.K. (2005) A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. *PLoS Genet.*, **1** (2), e26.

10 Liu, Q. *et al.* (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*, **10** (8), 1391–1406.

11 Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA*, **94** (3), 1035–1040.

12 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2005) Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci.*, **10** (2), 88–94.

13 Jaglo-Ottosen, K.R. *et al.* (1998) *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science*, **280** (5360), 104–106.

14 Choi, D.W., Rodriguez, E.M., and Close, T.J. (2002) Barley Cbf3 gene identification, expression pattern, and map location. *Plant Physiol.*, **129** (4), 1781–1787.

15 Jaglo, K.R. *et al.* (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.*, **127** (3), 910–917.

16 Dubouzet, J.G. *et al.* (2003) OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J.*, **33** (4), 751–763.

17 Qin, F. *et al.* (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. *Plant Cell Physiol.*, **45** (8), 1042–1052.

18 Zhang, X. *et al.* (2004) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. *Plant J.*, **39** (6), 905–919.

19 Xin, Z. and Browse, J. (1998) Eskimo1 mutants of *Arabidopsis* are constitutively freezing-tolerant. *Proc. Natl. Acad. Sci. USA*, **95** (13), 7799–7804.

20 Xin, Z. *et al.* (2007) *Arabidopsis* ESK1 encodes a novel regulator of freezing tolerance. *Plant J.*, **49** (5), 786–799.

21 Ishitani, M. *et al.* (1997) Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell*, **9** (11), 1935–1949.

22 Zhu, J. *et al.* (2004) An *Arabidopsis* homeodomain transcription factor gene, HOS9, mediates cold tolerance through a CBF-independent pathway. *Proc. Natl. Acad. Sci. USA*, **101** (26), 9873–9878.

23 Klingler, J.P., Batelli, G., and Zhu, J.K. (2010) ABA receptors: the START of a new paradigm in phytohormone signalling. *J. Exp. Bot.*, **61** (12), 3199–3210.

24 Raghavendra, A.S. *et al.* (2010) ABA perception and signalling. *Trends Plant Sci.*, **15** (7), 395–401.

25 Xiong, L. *et al.* (2002) Repression of stress-responsive genes by FIERY2, a novel transcriptional regulator in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **99** (16), 10899–10904.

26 Koornneef, M. *et al.* (1998) The genetic and molecular dissection of abscisic acid biosynthesis and signal transduction in *Arabidopsis*. *Plant Physiol. Biochem.*, **36**, 83–89.

27 Taylor, I.B., Burbidge, A., and Thompson, A.J. (2000) Control of abscisic acid synthesis. *J. Exp. Bot.*, **51** (350), 1563–1574.

28 Mantyla, E., Lang, V., and Palva, E.T., (1995) Role of Abscisic Acid in Drought-Induced Freezing Tolerance, Cold Acclimation, and Accumulation of LT178 and RAB18 Proteins in Arabidopsis thaliana. *Plant Physiol.*, **107** (1), 141–148.

29 Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.*, **2**, 48–54.

30 Ingram, J. and Bartel, D. (1996) The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 377–403.

31 Rock, C.D. (2000) Pathways to abscisic acid-regulated gene expression. *New Phytol.*, **148**, 357–396.

32 Choi, H. *et al.* (2000) ABFs, a family of ABA-responsive element binding factors. *J. Biol. Chem.*, **275** (3), 1723–1730.

33 Uno, Y. *et al.* (2000) *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc. Natl. Acad. Sci. USA*, **97** (21), 11632–11637.

34 Chinnusamy, V. *et al.* (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.*, **17** (8), 1043–1054.

35 Zarka, D.G. *et al.* (2003) Cold induction of *Arabidopsis* CBF genes involves multiple ICE (inducer of CBF expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiol.*, **133** (2), 910–918.

36 Agarwal, M. *et al.* (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J. Biol. Chem.*, **281** (49), 37636–37645.

37 Vogel, J.T. *et al.* (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *Plant J.*, **41** (2), 195–211.

38 Novillo, F. *et al.* (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **101** (11), 3985–3990.

39 Zhu, J., Dong, C.H., and Zhu, J.K. (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr. Opin. Plant Biol.*, **10** (3), 290–295.

40 Lee, B.H. *et al.* (2006) STABILIZED1, a stress-upregulated nuclear protein, is required for pre-mRNA splicing, mRNA turnover, and stress tolerance in *Arabidopsis*. *Plant Cell*, **18** (7), 1736–1749.

41 Dong, C.H. *et al.* (2006) A putative *Arabidopsis* nucleoporin, AtNUP160, is critical for RNA export and required for plant tolerance to cold stress. *Mol. Cell Biol.*, **26** (24), 9533–9543.

42 Gong, Z. *et al.* (2005) A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in *Arabidopsis*. *Plant Cell*, **17** (1), 256–267.

43 Gong, Z. *et al.* (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc. Natl. Acad. Sci. USA*, **99** (17), 11507–11512.

44 Koiwa, H. *et al.* (2002) C-terminal domain phosphatase-like family members (AtCPLs) differentially regulate *Arabidopsis thaliana* abiotic stress signaling, growth, and development. *Proc. Natl. Acad. Sci. USA*, **99** (16), 10893–10898.

45 Aukerman, M.J. and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell*, **15** (11), 2730–2741.

46 Brodersen, P. *et al.* (2008) Widespread translational inhibition by plant miRNAs and siRNAs. *Science*, **320** (5880), 1185–1190.

47 Chen, X. (2004) A microRNA as a translational repressor of APETALA2 in *Arabidopsis* flower development. *Science*, **303** (5666), 2022–2025.

48 Fahlgren, N. *et al.* (2010) MicroRNA gene evolution in *Arabidopsis lyrata* and *Arabidopsis thaliana*. *Plant Cell*, **22** (4), 1074–1089.

49 Khraiwesh, B. *et al.* (2010) Transcriptional control of gene expression by microRNAs. *Cell*, **140** (1), 111–122.

50 Llave, C. *et al.* (2002) Cleavage of scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science*, **297** (5589), 2053–2056.

51 Xie, Z., Kasschau, K.D., and Carrington, J.C. (2003) Negative feedback regulation of Dicer-Like1 in *Arabidopsis* by microRNA-guided mRNA degradation. *Curr. Biol.*, **13** (9), 784–789.

52 Mitsuda, N. and Ohme-Takagi, M. (2009) Functional analysis of transcription factors in *Arabidopsis*. *Plant Cell Physiol.*, **50** (7), 1232–1248.

53 Rhoades, M.W. *et al.* (2002) Prediction of plant microRNA targets. *Cell*, **110** (4), 513–520.

54 Jones-Rhoades, M.W. and Bartel, D. (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell*, **14** (6), 787–799.

55 Palatnik, J.F. *et al.* (2003) Control of leaf morphogenesis by microRNAs. *Nature*, **425** (6955), 257–263.

56 Covarrubias, A.A. and Reyes, J.L. (2010) Post-transcriptional gene regulation of salinity and drought responses by plant microRNAs. *Plant Cell Environ.*, **33** (4), 481–489.

57 Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell*, **16** (8), 2001–2019.

58 Zhou, X. *et al.* (2008) Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochim. Biophys. Acta*, **1779** (11), 780–788.

59 Liu, H.H. *et al.* (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA*, **14** (5), 836–843.

60 Lv, D.K. *et al.* (2010) Profiling of cold-stress-responsive miRNAs in rice by microarrays. *Gene*, **459** (1–2), 39–47.

61 Zhang, J. *et al.* (2009) Deep sequencing of *Brachypodium* small RNAs at the global genome level identifies microRNAs involved in cold stress response. *BMC Genomics*, **10**, 449.

62 Zhang, L. *et al.* (2009) A genome-wide characterization of microRNA genes in maize. *PLoS Genet.*, **5** (11), e1000716.

63 Jeong, D.H. *et al.* (2010) Abiotic stress-associated miRNAs: detection and functional analysis. *Methods Mol. Biol.*, **592**, 203–230.

64 Dong, C.H. *et al.* (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proc. Natl. Acad. Sci. USA*, **103** (21), 8281–8286.

65 Smalle, J. and Vierstra, R.D. (2004) The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol.*, **55**, 555–590.

66 Miura, K. *et al.* (2007) SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in *Arabidopsis*. *Plant Cell*, **19** (4), 1403–1414.

67 Miura, K., Jin, J.B., and Hasegawa, P.M. (2007) Sumoylation: a post-translational regulatory process in plants. *Curr. Opin. Plant Biol.*, **10** (5), 495–502.

68 Duan, K. *et al.* (2008) Characterization of a sub-family of *Arabidopsis* genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. *Plant J.*, **54** (6), 965–975.

69 Miura, K. *et al.* (2005) The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proc. Natl. Acad. Sci. USA*, **102** (21), 7760–7765.

70 Mikami, K. *et al.* (1998) A gene encoding phosphatidylinositol-4-phosphate 5-kinase is induced by water stress and abscisic acid in *Arabidopsis thaliana*. *Plant J.*, **15** (4), 563–568.

71 Tasma, I.M. *et al.* (2008) Expression and evolution of the phosphoinositide-specific phospholipase C gene family in *Arabidopsis thaliana*. *Plant Physiol. Biochem.*, **46** (7), 627–637.

72 Hirayama, T. *et al.* (1995) A gene encoding a phosphatidylinositol-specific phospholipase C is induced by

dehydration and salt stress in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, **92** (9), 3903–3907.
73 Sanchez, J. and Chua, N.H. (2001) *Arabidopsis* PLC1 is required for secondary responses to abscisic acid signals. *Plant Cell*, **13** (5), 1143–1154.
74 Sanders, D., Brownlee, C., and Harper, J.F. (1999) Communicating with calcium. *Plant Cell*, **11** (4), 691–706.
75 Schroeder, J.I. *et al.* (2001) Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **52**, 627–658.
76 Bargmann, B.O. and Munnik, T. (2006) The role of phospholipase D in plant stress responses. *Curr. Opin. Plant Biol.*, **9** (5), 515–522.
77 Wang, X. (1999) The role of phospholipase D in signaling cascades. *Plant Physiol.*, **120** (3), 645–652.
78 Li, W. *et al.* (2004) The plasma membrane-bound phospholipase Ddelta enhances freezing tolerance in *Arabidopsis thaliana*. *Nat. Biotechnol.*, **22** (4), 427–433.
79 Ruelland, E. *et al.* (2002) Activation of phospholipases C and D is an early response to a cold exposure in *Arabidopsis* suspension cells. *Plant Physiol.*, **130** (2), 999–1007.
80 Vergnolle, C. *et al.* (2005) The cold-induced early activation of phospholipase C and D pathways determines the response of two distinct clusters of genes in *Arabidopsis* cell suspensions. *Plant Physiol.*, **139** (3), 1217–1233.
81 Tuteja, N. and Sopory, S.K. (2008) Plant signaling in stress: G-protein coupled receptors, heterotrimeric G-proteins and signal coupling via phospholipases. *Plant Signal. Behav.*, **3** (2), 79–86.
82 Xiong, L. *et al.* (2001) FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in *Arabidopsis*. *Genes Dev.*, **15** (15), 1971–1984.
83 Gill, S.S. and Tuteja, N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.*, **48** (12), 909–930.
84 Miller, G. *et al.* (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.*, **33** (4), 453–467.
85 Takahashi, S. and Murata, N. (2008) How do environmental stresses accelerate photoinhibition? *Trends Plant Sci.*, **13** (4), 178–182.
86 Huang, C. *et al.* (2005) Increased sensitivity to salt stress in an ascorbate-deficient *Arabidopsis* mutant. *J. Exp. Bot.*, **56** (422), 3041–3049.
87 Miao, Y. *et al.* (2006) An *Arabidopsis* glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. *Plant Cell*, **18** (10), 2749–2766.
88 Pnueli, L. *et al.* (2003) Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (Apx1)-deficient *Arabidopsis* plants. *Plant J.*, **34** (2), 187–203.
89 Davletova, S. *et al.* (2005) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. *Plant Cell*, **17** (1), 268–281.
90 Yuasa, T. *et al.* (2001) Oxidative stress activates ATMPK6: an *Arabidopsis* homologue of MAP kinase. *Plant Cell Physiol.*, **42** (9), 1012–1016.
91 Cheng, C. *et al.* (2007) An early response regulatory cluster induced by low temperature and hydrogen peroxide in seedlings of chilling-tolerant *Japonica* rice. *BMC Genomics*, **8**, 175.
92 Chen, W. and Singh, K.B. (1999) The auxin, hydrogen peroxide and salicylic acid induced expression of the *Arabidopsis* GST6 promoter is mediated in part by an ocs element. *Plant J.*, **19** (6), 667–677.
93 Wang, H. *et al.* (2004) Ectopic overexpression of tomato JERF3 in tobacco activates downstream gene expression and enhances salt tolerance. *Plant Mol. Biol.*, **55** (2), 183–192.
94 Wu, L. *et al.* (2008) Transcriptional modulation of ethylene response factor protein JERF3 in the oxidative stress response enhances tolerance of tobacco seedlings to salt, drought, and freezing. *Plant Physiol.*, **148** (4), 1953–1963.

95 Rizhsky, L. *et al.* (2004) The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. *J. Biol. Chem.*, **279** (12), 11736–11743.

96 Davletova, S. *et al.* (2005) The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in *Arabidopsis*. *Plant Physiol.*, **139** (2), 847–856.

97 Ciftci-Yilmaz, S. *et al.* (2007) The EAR-motif of the Cys2/His2-type zinc finger protein Zat7 plays a key role in the defense response of *Arabidopsis* to salinity stress. *J. Biol. Chem.*, **282** (12), 9260–9268.

98 Knight, H. (2000) Calcium signaling during abiotic stress in plants. *Int. Rev. Cytol.*, **195**, 269–324.

99 Dodd, A.N., Kudla, J., and Sanders, D. (2010) The language of calcium signaling. *Annu. Rev. Plant Biol.*, **61**, 593–620.

100 Kudla, J., Batistic, O., and Hashimoto, K. (2010) Calcium signals: the lead currency of plant information processing. *Plant Cell*, **22** (3), 541–563.

101 Tuteja, N. and Mahajan, S. (2007) Calcium signaling network in plants: an overview. *Plant Signal Behav.*, **2** (2), 79–85.

102 DeFalco, T.A., Bender, K.W., and Snedden, W.A. (2010) Breaking the code: Ca^{2+} sensors in plant signalling. *Biochem. J.*, **425** (1), 27–40.

103 Luan, S. *et al.* (2002) Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. *Plant Cell* (14 Suppl), S389–S390.

104 Kim, M.C. *et al.* (2009) Calcium and calmodulin-mediated regulation of gene expression in plants. *Mol. Plant*, **2** (1), 13–21.

105 Boonburapong, B. and Buaboocha, T. (2007) Genome-wide identification and analyses of the rice calmodulin and related potential calcium sensor proteins. *BMC Plant Biol.*, **7**, 4.

106 McCormack, E., Tsai, Y.C., and Braam, J. (2005) Handling calcium signaling: *Arabidopsis* CaMs and CMLs. *Trends Plant Sci.*, **10** (8), 383–389.

107 Delk, N.A. *et al.* (2005) CML24, regulated in expression by diverse stimuli, encodes a potential Ca^{2+} sensor that functions in responses to abscisic acid, daylength, and ion stress. *Plant Physiol.*, **139** (1), 240–253.

108 Magnan, F. *et al.* (2008) Mutations in AtCML9, a calmodulin-like protein from *Arabidopsis thaliana*, alter plant responses to abiotic stress and abscisic acid. *Plant J.*, **56** (4), 575–589.

109 Das, R. and Pandey, G.K. (2010) Expressional analysis and role of calcium regulated kinases in abiotic stress signaling. *Curr. Genomics*, **11** (1), 2–13.

110 Ludwig, A.A., Romeis, T., and Jones, J.D. (2004) CDPK-mediated signalling pathways: specificity and cross-talk. *J. Exp. Bot.*, **55** (395), 181–188.

111 Martin, M.L. and Busconi, L. (2001) A rice membrane-bound calcium-dependent protein kinase is activated in response to low temperature. *Plant Physiol.*, **125** (3), 1442–1449.

112 Saijo, Y. *et al.* (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J.*, **23** (3), 319–327.

113 Saijo, Y. *et al.* (2001) A $Ca(^{2+})$-dependent protein kinase that endows rice plants with cold- and salt-stress tolerance functions in vascular bundles. *Plant Cell Physiol.*, **42** (11), 1228–1233.

114 Choi, H.I. *et al.* (2005) *Arabidopsis* calcium-dependent protein kinase AtCPK32 interacts with ABF4, a transcriptional regulator of abscisic acid-responsive gene expression, and modulates its activity. *Plant Physiol.*, **139** (4), 1750–1761.

115 Milla, M.A. *et al.* (2006) The *Arabidopsis* AtDi19 gene family encodes a novel type of Cys2/His2 zinc-finger protein implicated in ABA-independent dehydration, high-salinity stress and light signaling pathways. *Plant Mol. Biol.*, **61** (1–2), 13–30.

116 Rodriguez Milla, M.A. *et al.* (2006) A novel yeast two-hybrid approach to identify CDPK substrates: characterization of the interaction

between AtCDPK11 and AtDi19, a nuclear zinc finger protein. *FEBS Lett.*, **580**, 904–911.

117 Mehlmer, N. *et al.* (2010) The $Ca^{(2+)}$-dependent protein kinase CPK3 is required for MAPK-independent salt-stress acclimation in *Arabidopsis*. *Plant J.*, **63**, 484–498.

118 Albrecht, V. *et al.* (2003) The calcium sensor CBL1 integrates plant responses to abiotic stresses. *Plant J.*, **36** (4), 457–470.

119 Batistic, O. and Kudla, J. (2009) Plant calcineurin B-like proteins and their interacting protein kinases. *Biochim. Biophys. Acta*, **1793** (6), 985–992.

120 Cheong, Y.H. *et al.* (2003) CBL1: a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. *Plant Cell*, **15** (8), 1833–1845.

121 Kudla, J. *et al.* (1999) Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. *Proc. Natl. Acad. Sci. USA*, **96** (8), 4718–4723.

122 Pandey, G.K. *et al.* (2004) The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. *Plant Cell*, **16** (7), 1912–1924.

123 Shi, J. *et al.* (1999) Novel protein kinases associated with calcineurin B-like calcium sensors in *Arabidopsis*. *Plant Cell*, **11** (12), 2393–2405.

124 Kim, K.N. *et al.* (2000) Interaction specificity of *Arabidopsis* calcineurin B-like calcium sensors and their target kinases. *Plant Physiol.*, **124** (4), 1844–1853.

125 Luan, S. (2009) The CBL-CIPK network in plant calcium signaling. *Trends Plant Sci.*, **14** (1), 37–42.

126 Batistic, O. and Kudla, J. (2004) Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. *Planta*, **219** (6), 915–924.

127 Kolukisaoglu, U. *et al.* (2004) Calcium sensors and their interacting protein kinases: genomics of the *Arabidopsis* and rice CBL-CIPK signaling networks. *Plant Physiol.*, **134** (1), 43–58.

128 D'Angelo, C. *et al.* (2006) Alternative complex formation of the Ca-regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in *Arabidopsis*. *Plant J.*, **48** (6), 857–872.

129 Cheong, Y.H. *et al.* (2007) Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in *Arabidopsis*. *Plant J.*, **52** (2), 223–239.

130 Hedrich, R. and Kudla, J. (2006) Calcium signaling networks channel plant K+ uptake. *Cell*, **125** (7), 1221–1223.

131 Lee, S.C. *et al.* (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. *Proc. Natl. Acad. Sci. USA*, **104** (40), 15959–15964.

132 Li, L. *et al.* (2006) A Ca(2)+ signaling pathway regulates a K(+) channel for low-K response in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **103** (33), 12625–12630.

133 Kim, B.G. *et al.* (2007) The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in *Arabidopsis*. *Plant J.*, **52** (3), 473–484.

134 Ishitani, M. *et al.* (2000) SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. *Plant Cell*, **12** (9), 1667–1678.

135 Liu, J. and Zhu, J.K. (1997) An *Arabidopsis* mutant that requires increased calcium for potassium nutrition and salt tolerance. *Proc. Natl. Acad. Sci. USA*, **94** (26), 14960–14964.

136 Liu, J. and Zhu, J.K. (1998) A calcium sensor homolog required for plant salt tolerance. *Science*, **280** (5371), 1943–1945.

137 Zhu, J.K. (2000) Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiol.*, **124** (3), 941–948.

138 Zhu, J.K., Liu, J., and Xiong, L. (1998) Genetic analysis of salt tolerance in *Arabidopsis*. Evidence for a critical role of potassium nutrition. *Plant Cell*, **10** (7), 1181–1191.

139 Guo, Y. *et al.* (2001) Molecular characterization of functional domains in the protein kinase SOS2 that is required

for plant salt tolerance. *Plant Cell*, **13** (6), 1383–1400.

140 Liu, J. *et al.* (2000) The *Arabidopsis thaliana* SOS2 gene encodes a protein kinase that is required for salt tolerance. *Proc. Natl. Acad. Sci. USA*, **97** (7), 3730–3734.

141 Halfter, U., Ishitani, M., and Zhu, J.K. (2000) The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. *Proc. Natl. Acad. Sci. USA*, **97** (7), 3735–3740.

142 Shi, H. *et al.* (2000) The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na + /H + antiporter. *Proc. Natl. Acad. Sci. USA*, **97** (12), 6896–6901.

143 Qiu, Q.S. *et al.* (2002) Regulation of SOS1, a plasma membrane Na + /H + exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proc. Natl. Acad. Sci. USA*, **99** (12), 8436–8441.

144 Batistic, O. *et al.* (2010) CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. *Plant J.*, **61** (2), 211–222.

145 Hunter, T. (1995) When is a lipid kinase not a lipid kinase? When it is a protein kinase. *Cell*, **83** (1), 1–4.

146 Schwartz, M.A. and Madhani, H.D. (2004) Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.*, **38**, 725–748.

147 Andreasson, E. and Ellis, B. (2010) Convergence and specificity in the *Arabidopsis* MAPK nexus. *Trends Plant. Sci.*, **15** (2), 106–113.

148 Ichimura, K. *et al.* (1998) Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in *Arabidopsis*. *Biochem. Biophys. Res. Commun.*, **253** (2), 532–543.

149 Mishra, N.S., Tuteja, R., and Tuteja, N. (2006) Signaling through MAP kinase networks in plants. *Arch. Biochem. Biophys.*, **452** (1), 55–68.

150 Mizoguchi, T. *et al.* (1998) Identification of a possible MAP kinase cascade in *Arabidopsis thaliana* based on pairwise yeast two-hybrid analysis and functional complementation tests of yeast mutants. *FEBS Lett.*, **437** (1–2), 56–60.

151 Asai, T. *et al.* (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature*, **415** (6875), 977–983.

152 Teige, M. *et al.* (2004) The MKK2 pathway mediates cold and salt stress signaling in *Arabidopsis*. *Mol. Cell*, **15** (1), 141–152.

153 Ichimura, K. *et al.* (2000) Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. *Plant J.*, **24** (5), 655–665.

154 Mizoguchi, T. *et al.* (1996) A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, **93** (2), 765–769.

155 Petersen, M. *et al.* (2000) *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. *Cell*, **103** (7), 1111–1120.

156 Droillard, M.J. *et al.* (2004) Involvement of MPK4 in osmotic stress response pathways in cell suspensions and plantlets of *Arabidopsis thaliana*: activation by hypoosmolarity and negative role in hyperosmolarity tolerance. *FEBS Lett.*, **574** (1–3), 42–48.

157 Xiong, L. and Yang, Y. (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell*, **15** (3), 745–759.

158 Andreasson, E. *et al.* (2005) The MAP kinase substrate MKS1 is a regulator of plant defense responses. *EMBO J.*, **24** (14), 2579–2589.

159 Feilner, T. *et al.* (2005) High throughput identification of potential *Arabidopsis* mitogen-activated protein kinases substrates. *Mol. Cell Proteomics*, **4** (10), 1558–1568.

160 Yoshiki, S. *et al.* (2010) Ras and calcium signaling pathways converge at Raf1 via the Shoc2 scaffold protein. *Mol. Biol. Cell*, **21** (6), 1088–1096.

161 Bartels, S. *et al.* (2010) Emerging functions for plant MAP kinase phosphatases. *Trends Plant Sci.*, **15** (6), 322–329.

162 Ulm, R. *et al.* (2002) Distinct regulation of salinity and genotoxic stress responses by *Arabidopsis* MAP kinase phosphatase 1. *EMBO J.*, **21** (23), 6483–6493.

163 Schweighofer, A. *et al.* (2007) The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in *Arabidopsis*. *Plant Cell*, **19** (7), 2213–2224.

164 Xu, Q. *et al.* (1998) Molecular characterization of a tyrosine-specific protein phosphatase encoded by a stress-responsive gene in *Arabidopsis*. *Plant Cell*, **10** (5), 849–857.

165 Bartels, S. *et al.* (2009) MAP kinase phosphatase1 and protein tyrosine phosphatase1 are repressors of salicylic acid synthesis and SNC1-mediated responses in *Arabidopsis*. *Plant Cell*, **21** (9), 2884–2897.

166 Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000) Two-component signal transduction. *Annu. Rev. Biochem.*, **69**, 183–215.

167 Hwang, I., Chen, H.C., and Sheen, J. (2002) Two-component signal transduction pathways in *Arabidopsis*. *Plant Physiol.*, **129** (2), 500–515.

168 Koretke, K.K. *et al.* (2000) Evolution of two-component signal transduction. *Mol. Biol. Evol.*, **17** (12), 1956–1970.

169 Pareek, A. *et al.* (2006) Whole-genome analysis of *Oryza sativa* reveals similar architecture of two-component signaling machinery with *Arabidopsis*. *Plant Physiol.*, **142** (2), 380–397.

170 Pils, B. and Heyl, A. (2009) Unraveling the evolution of cytokinin signaling. *Plant Physiol.*, **151** (2), 782–791.

171 To, J. and Kieber, J.J. (2008) Cytokinin signaling: two-components and more. *Trends Plant Sci.*, **13** (2), 85–92.

172 Mochida, K. *et al.* (2010) Genome-wide analysis of two-component systems and prediction of stress-responsive two-component system members in soybean. *DNA Res.*, **17** (5), 303–324.

173 Tran, L.S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2010) Role of cytokinin responsive two-component system in ABA and osmotic stress signalings. *Plant Signal Behav.*, **5** (2), 148–150.

174 Urao, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2001) Plant histidine kinases: an emerging picture of two-component signal transduction in hormone and environmental responses. *Sci. STKE*, **2001** (109), re18.

175 Tran, L.S. *et al.* (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **104** (51), 20623–20628.

176 Jain, M., Tyagi, A.K., and Khurana, J. (2006) Molecular characterization and differential expression of cytokinin-responsive type-A response regulators in rice (*Oryza sativa*). *BMC Plant Biol.*, **6**, 1.

177 Perochon, A. *et al.* (2010) Interaction of a plant pseudo-response regulator with a calmodulin-like protein. *Biochem. Biophys. Res. Commun.*, **398** (4), 747–751.

178 Karan, R., Singla-Pareek, S.L., and Pareek, A. (2009) Histidine kinase and response regulator genes as they relate to salinity tolerance in rice. *Funct. Integr. Genomics*, **9** (3), 411–417.

179 Wilkie, T.M. and Yokoyama, S. (1994) Evolution of the G protein alpha subunit multigene family. *Soc. Gen. Physiol. Ser.*, **49**, 249–270.

180 Seack, J., Kruse, M., and Muller, W.E. (1998) Evolutionary analysis of G-proteins in early metazoans: cloning of alpha- and beta-subunits from the sponge *Geodia cydonium*. *Biochim. Biophys. Acta*, **1401** (1), 93–103.

181 Cook, L.A. *et al.* (2001) Identification of a region in G protein gamma subunits conserved across species but hypervariable among subunit isoforms. *Protein Sci.*, **10** (12), 2548–2555.

182 Ma, H., Yanofsky, M.F., and Meyerowitz, E.M. (1990) Molecular cloning and characterization of GPA1, a G protein alpha subunit gene from

Arabidopsis thaliana. *Proc. Natl. Acad. Sci. USA*, **87** (10), 3821–3825.

183 Weiss, C.A. *et al.* (1994) Isolation of cDNAs encoding guanine nucleotide-binding protein beta-subunit homologues from maize (ZGB1) and *Arabidopsis* (AGB1). *Proc. Natl. Acad. Sci. USA*, **91** (20), 9554–9558.

184 Initiative, A.G. (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408** (6814), 796–815.

185 Mason, M.G. and Botella, J.R. (2001) Isolation of a novel G-protein gamma-subunit from *Arabidopsis thaliana* and its interaction with Gbeta. *Biochim. Biophys. Acta*, **1520** (2), 147–153.

186 Assmann, S.M. (2002) Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. *Plant Cell* (14 Suppl), S355–S373.

187 Temple, B.R. and Jones, A.M. (2007) The plant heterotrimeric G-protein complex. *Annu. Rev. Plant Biol.*, **58**, 249–266.

188 Asakura, Y. and Kurosaki, F. (2007) Cloning and expression of Dcga gene encoding alpha subunit of GTP-binding protein in carrot seedlings. *Biol. Pharm. Bull.*, **30** (9), 1800–1804.

189 Misra, S. *et al.* (2007) Heterotrimeric G-protein complex and G-protein-coupled receptor from a legume (*Pisum sativum*): role in salinity and heat stress and cross-talk with phospholipase C. *Plant J.*, **51** (4), 656–669.

190 Gao, Y. *et al.* (2010) Isolation and characterization of gene encoding G protein alpha subunit protein responsive to plant hormones and abiotic stresses in *Brassica napus*. *Mol. Biol. Rep.*, **37** (8), 3957–3965.

191 Klein, S., Reuveni, H., and Levitzki, A. (2000) Signal transduction by a nondissociable heterotrimeric yeast G protein. *Proc. Natl. Acad. Sci. USA*, **97** (7), 3219–3223.

192 von Zastrow, M. and Mostov, K. (2001) Signal transduction. A new thread in an intricate web. *Science*, **294** (5548), 1845–1847.

193 Bockaert, J. and Pin, J. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.*, **18** (7), 1723–1729.

194 Colucci, G. *et al.* (2002) GCR1, the putative *Arabidopsis* G protein-coupled receptor gene is cell cycle-regulated, and its overexpression abolishes seed dormancy and shortens time to flowering. *Proc. Natl. Acad. Sci. USA*, **99** (7), 4736–4741.

195 Moriyama, E.N. *et al.* (2006) Mining the *Arabidopsis thaliana* genome for highly-divergent seven transmembrane receptors. *Genome Biol.*, **7** (10), R96.

196 Pandey, S., Nelson, D.C., and Assmann, S.M. (2009) Two novel GPCR-type G proteins are abscisic acid receptors in *Arabidopsis*. *Cell*, **136** (1), 136–148.

197 Lein, W. and Saalbach, G. (2001) Cloning and direct G-protein regulation of phospholipase D from tobacco. *Biochim. Biophys Acta*, **1530** (2–3), 172–183.

198 Zhao, J. and Wang, X. (2004) *Arabidopsis* phospholipase DALPHA1 interacts with the heterotrimeric G-protein alpha-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. *J. Biol. Chem.*, **279** (3), 1794–1800.

199 Mishra, G. *et al.* (2006) A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in *Arabidopsis*. *Science*, **312** (5771), 264–266.

200 Morillo, S.A. and Tax, F.E. (2006) Functional analysis of receptor-like kinases in monocots and dicots. *Curr. Opin. Plant Biol.*, **9** (5), 460–469.

201 Shiu, S.H., Karlowski, W.M., Pan, R., Tzeng, Y.H., Mayer, K.F., and Li, W.H. (2004) Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell*, **16** (5), 1220–1234.

202 Hong, S.W. *et al.* (1997) Identification of a receptor-like protein kinase gene rapidly induced by abscisic acid, dehydration, high salt, and cold treatments in *Arabidopsis thaliana*. *Plant Physiol.*, **113** (4), 1203–1212.

203 Osakabe, Y. *et al.* (2010) Overproduction of the membrane-bound receptor-like protein kinase 1, RPK1, enhances abiotic stress tolerance in *Arabidopsis*. *J. Biol. Chem.*, **285** (12), 9190–9201.

204 Chae, L. *et al.* (2009) Diverse transcriptional programs associated with environmental stress and hormones in the *Arabidopsis* receptor-like kinase gene family. *Mol. Plant*, **2** (1), 84–107.

205 Nühse, T.S. *et al.* (2004) Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. *Plant Cell*, **16** (9), 2394–2405.

6
Mechanism of Sulfur Dioxide Toxicity and Tolerance in Crop Plants

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Air is an important and vital resource both for the sustenance and for the development of every living organism. The composition of its minor constituents often varies as a result of the emission or contaminants from various activities. A huge amount of toxic materials and gas including SO_2 is released into the atmosphere originated from different kinds of industries and other human activities that eventually pollute the atmosphere. A mere change in the gaseous composition of the atmosphere has many different impacts on terrestrial plants. Sulfur dioxide pollution is known to have a substantial effect on agricultural production and is still of great significance in many developing countries. Conversely, due to strict regulatory control on SO_2 emissions, the level of atmospheric SO_2 in developed countries has radically declined causing S-deficiency symptoms in crop plants, resulting in a drastic reduction in crop productivity and quality. Increased uptake of SO_2 can impair plant metabolism leading to reduced growth and productivity due to accumulation of sulfite and sulfate within the plant. Phytotoxicity of SO_2 is determined by the environmental conditions, the duration of exposure, the atmospheric SO_2 concentration, the sulfur status of the soil, the genetic constitution of the plant, and the developmental phase of plants. Plants form a sink for atmospheric SO_2, which is taken up by the foliage. Since the internal (mesophyll) resistance to SO_2 is low due to its high solubility and rapid dissociation in the cell sap, foliar SO_2 uptake is determined by its diffusion through the stomata. Foliar injury may be caused by the negative effects of acidification of tissue/cells after the dissociation of the absorbed SO_2 and the reaction of the formed sulfite with cellular components. There is also a wide inter- and intraspecific variation in susceptibility between species; however, the physiological basis for the variation in air pollution response is still largely unresolved. Paradoxically, atmospheric SO_2 may also be used as plant nutrient where SO_2 absorbed by the leave can enter the S assimilatory pathway directly or after oxidation to SO_4^{2-} and be reduced to sulfide, incorporated into cysteine and subsequently, organic S compounds, and utilized as S nutrient. Plants may also benefit from SO_2 exposure given that it can contribute to the plants' S nutrition, and result in enhanced crop productivity, especially in plants growing in sulfur-deficient soils.

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6.1 Introduction

Sulfur dioxide (SO_2) a colorless, nonflammable gas is one of the most prevalent phytotoxic gaseous pollutants released as a result of combustion of fossil fuels in developing countries including India [1–3] and causes disorders in plants with specific symptoms [4, 5]. However, SO_2 at low concentration can stimulate physiology and growth of plants, especially in plants growing in sulfur-deficient soil [6], where the sulfate might be metabolized to fulfill the demand for sulfur as a nutrient [7]. Sulfur is necessary for proper growth and development of living organisms; however, it is attributed rather catalytic and regulatory than structural functions because it is much less abundant than other macroelements. The plant biomass consumed as food and feed serves as the main source of organic sulfur for animals and humans [8]. Plants, bacteria, and fungi, unlike animals, are able to assimilate inorganic sulfur and incorporate it into organic compounds. Plants utilize sulfate for the synthesis of diverse primary and secondary metabolites [9]. However, increased uptake of SO_2, causes toxicity and reduces growth and productivity of plants due to accumulation of sulfite or sulfate ions in excess [6, 10, 11].

6.2 Emission Sources

6.2.1 Natural Sources

Natural sources of sulfur dioxide include volcanoes and volcanic vents, microbial activities (decaying organic matter), solar action on seawater, and oxidation of dimethyl sulfide emitted from the ocean [12]. According to Hazardous Substances Data Bank (HSDB) [13], although volcanoes are a sporadic source of sulfur dioxide, they are potentially a significant natural source. Decaying organic matter indirectly results in a natural source of SO_2. Decaying organic matter on land, in marshes, and in oceans results in the release of hydrogen sulfide, which is quickly oxidized to SO_2.

6.2.2 Anthropogenic Sources

On a global scale, anthropogenic emissions represent a significant contribution to the SO_2 emitted to the atmosphere [14], and these emissions are approximately equal to natural emissions [15]. Friend [16] estimates that, on a global basis, 75–85% of SO_2 emissions are the result of fossil fuel combustion, while the remainder of the emissions is the result of refining and smelting. It is estimated that approximately 93% of the global SO_2 emissions occur in the northern

hemisphere and the remaining 7% in the southern hemisphere [14]. The greatest anthropogenic sources of SO_2 result from the combustion of fossil fuels and from the smelting sulfide ores [12]. Another significant source is petroleum refining [13]. Other less significant sources include chemical and allied products manufacturing, metal processing, other industrial processes, and vehicle emissions [17].

Together, natural and anthropogenic sources emit an estimated 194 million ton of SO_2 per annum, of which 83% is due to fossil fuel combustion [18]. Although considerable progress has been made in the development and implementation of SO_2 control technologies in North America, Europe, and Japan, ambient SO_2 concentrations is still a significant problem in many parts of the world particularly the developing countries including India [19–21]. Nearly two-thirds of the total mined coal is burnt in thermal power stations to generate electricity in India and other developing countries. Coal combustion liberates high concentrations of oxides of sulfur and nitrogen into the environment. The ambient concentration of SO_2 varies with the distance from the source and direction of wind. The surrounding environment of approximately 10–20 km diameter may experience much higher concentrations of SO_2. Khan and Khan [22, 23] recorded 169–298 μg SO_2 m^{-3} at a site 2 km away from a coal-fired power plant in the usual wind direction. According to CPCB, New Delhi, permissible level of SO_2 for agricultural areas is 50 μg m^{-3} for annual and 80 μg m^{-3} for 24 h means (see Table 6.1). Once emitted, SO_2 is transferred from the atmosphere to surfaces by diffusion (both dry and wet deposition) at variable rates that are strongly influenced by meteorological conditions. It is also important to note that SO_2 in the atmosphere is also transformed to SO_4^{2-} at variable rates, and these SO_4^{2-} particles are deposited on surfaces by Brownian motion (dry deposition) and by precipitation (wet deposition). Any observed foliar injury or changes in plant growth and productivity due to SO_2 exposures are the result of dry/wet deposition and subsequent uptake of sulfate and sulfite ions in the leaf tissue and their uptake by plants [24].

Table 6.1 National ambient quality standard for sulfur dioxide (SO_2).

	SO_2 (μg m^{-3})	
Time-weighed average	**Annual**	**24 h**
Industrial, residential, rural and other areas	50	80
Ecologically sensitive areas (notified by Govt. of India)	20	80
Methods of measurement	Improved West and Gaeke	Ultraviolet fluorescence

Source: Central Pollution Control Board, 2009, India.

6.3 Effects on Plants

6.3.1 Visible Foliar Injury

As leaves are more sensitive to SO_2 exposure than stems, buds, and reproductive parts, SO_2 injury to plants is first seen on the foliage. The degree to which foliage responds to SO_2 is generally determined by both biotic (genetic makeup, developmental stage of growth, plant nutrient status, and pests and diseases) and abiotic factors (soil moisture and nutrient status, air and soil temperature, relative humidity, radiation, precipitation, and metrological conditions, as well as the presence of other air pollutants), as well as by the concentration, duration, and frequency of SO_2 exposure. Basically, when biotic and abiotic factors are favorable for plant growth and development, there is a high probability that plants exposed to SO_2 will be adversely affected. When one or more biotic and/or abiotic factors limit a plant's growth and development, there is a lowered probability that the plant will be adversely affected. Besides, SO_2 concentration, as well as duration and frequency of SO_2 exposure that are determined by meteorological conditions, play a significant role in determining the potential for an adverse response of vegetation to SO_2 [25].

Sulfur dioxide when present at higher concentrations causes foliar injury in plants. It diffuses in plants through open stomata and reacts with moisture to produce sulfite ions [24]. If the formation of sulfite ions is slow, they are oxidized to sulfate ions and utilized by plants. However, excess accumulation of sulfite and sulfate ions is toxic to plants [26]. The sulfite ions are about 30 times more toxic than sulfate ions [27]. Generally, two types of markers or symptoms designated as acute and chronic are produced by plants due to the accumulation of sulfite ions in the leaf tissue. An acute SO_2 exposure is considered as a short duration SO_2 exposure (from minutes to hours) that is of sufficient concentration to result in the expression of necrotic injury to the foliage within a few hours or days. These acute SO_2 injury symptoms commonly consist of bifacial, marginal, and/or interveinal necrosis and chlorosis on leaves at the full stage of development on broad-leaved plants. The necrotic areas can range from white to reddish brown to black in color depending on the plant species, and the margins of the necrotic areas are mostly irregular and occasionally dark pigmented. In monocotyledonous plants, acute injury symptoms start at the tip of the leaves and spread downward as necrotic and chlorotic streaks with occasional reddish pigmentation. In coniferous plants, acute symptoms appear on second-year or older needles and consist of a tan-to-reddish brown necrosis that starts at the needle tips, spreads downward toward the base, and is commonly preceded by chlorosis [25]. In case of severe injury, abscission layer develops at the base of petiole and the leaves fall down [28]. Although the above acute foliar SO_2 injury symptoms are standard/accepted symptoms of acute SO_2 exposure, very similar injury symptoms can also be caused by other air pollutants and biotic and abiotic stress factors [29]. When leaf surfaces are wet at the time of exposure, SO_2 can be absorbed by the water droplets on the leaf surface and the water becomes acidic as the SO_2 is converted into sulfuric acid

(H_2SO_2) and can cause acute foliar injury. Acute foliar injury from this "acidic wet deposition" occurs as necrotic areas with regular margins that reflect the outline of the acidified water droplets on the leaf surface. These short-term, acute foliar injury symptoms may or may not lead to long-term reductions in plant growth and productivity [30]. Low concentration SO_2 exposures that occur during the entire growth cycle or life of a plant with periodic intermittent and random peak levels is considered as chronic SO_2 exposure [31]. Such exposures may or may not result in chronic foliar injury symptoms such as marginal and/or interveinal chlorosis in broad-leaved plants, chlorosis in second-year and older conifer needles, premature fall coloration, and premature leaf/needle abscission [29]. In the same way as in the case of acute foliar injury symptoms, symptoms similar to chronic injury can be caused by other air pollutants and biotic and abiotic stress factors. Chronic SO_2 exposures can lead to reduction in the rate of plant growth and productivity (biomass), for example, grasses [32] and pines [33]. It is important to note that the reduction in plant growth and productivity from chronic exposure may occur without any development of visible chronic foliar injury symptoms. Physiological, biochemical, cellular, and tissue-level markers can be used to identify the presence of chronic SO_2 stress when visible symptoms are present or absent [34]. It is also important to mention that in the vicinity of point source, acute SO_2 exposures can occur on top of chronic SO_2 exposures. Furthermore, depending on the SO_2 concentration, duration, and frequency, acute and chronic SO_2 injury symptoms can cooccur on the same or different foliage of the same plant [25]. The expression of acute and/or chronic SO_2 symptoms is highly variable and can vary at the genus, species, variety or cultivar, provenance, and population levels [35, 36].

Shaw *et al.* [37] reported the effects of 34 and 58 $\mu g\ SO_2\ m^{-3}$ on needle necrosis in Scots pine (*Pinus sylvestris* L.) during the fumigation period of the Liphook Forest Fumigation Project. Regression analysis showed that the appearance of foliar injury was related to the mean SO_2 concentration during a critical growth period although injury did not become visible until 5 weeks later. SO_2 at 58 $\mu g\ m^{-3}$ caused foliar injury to a greater number of trees in 2 of the 3 survey years and foliar injury appeared on the same trees in consecutive years suggesting that the sensitivity was genetic. Furthermore, to see if the injury symptoms observed in the field could be duplicated, a subsidiary fumigation chamber experiment was performed. The result revealed that exposure to 655, 1310, and 2619 $\mu g\ m^{-3}$ of SO_2 for 4 h on Scots pine seedlings produced no effect in any treatment suggesting that this may have been due to a low replicate number resulting in a few plants at the most sensitive stage of growth and/or due to low humidity during fumigation. Intermittent exposure of tomato (cv. Pusa Ruby) to SO_2 at 286 $\mu g\ m^{-3}$ (3 h every third day for 75 days) induced slight chlorosis in leaves; however, considerable chlorosis with browning developed on the foliage at 571 $\mu g\ m^{-3}$ of SO_2. Symptoms were more pronounced and appeared earlier on SO_2-exposed plants infected with *Meloidogyne incognita* race 1 especially in post- and concomitant inoculation exposure [1]. Clapperton and Reid [38] screened genotypes of timothy (*Phleum pratense*) for SO_2 sensitivity in experiments conducted in closed fumigation chambers. Plants exposed to 393–524 $\mu g\ m^{-3}$ of SO_2 for 3 weeks developed chlorotic areas, browning, and necrosis of the leaves. Foliar and flower

injury occurred in *Calendula officianalis* [39] and *Zinnia* [40], and the intensity of symptoms increased with SO_2 concentration and duration of exposure. Rakwal *et al.* [41] observed distinctive reddish brown necrotic spots and interveinal browning appeared on the leaf surface of rice seedling cv. Nipponbare after exposure to SO_2 over control, partly reminiscent of the hypersensitive reaction lesions. Intermittent exposure to SO_2 at 200 and 300 $\mu g\, m^{-3}$ caused chlorosis of the leaves of pumpkin with or without inoculation of *M. javanica*. Only a mild chlorosis appeared in the infected plants at 100 $\mu g\, m^{-3}$ of SO_2 [42]. Sulfur dioxide (0.1 ppm) induced foliar chlorosis on two cultivars of cowpea, namely, V-38-1 and V-218, which appeared earlier in the presence of root-knot nematode (*M. incognita*) [43].

6.3.2 Sulfur Uptake and Plant Sulfur Content

Sulfur is prominently taken up by the roots in the form of sulfate ions. Sulfate is then transported to the shoot through xylem, where it gets reduced in the chloroplast prior to its assimilation into organic sulfur compounds. Sulfate is activated by ATP to APS (adenosine 5′ phosphosulfate), catalyzed by TP sulfurylase, and subsequently reduced by APS reductase to sulfite and then by sulfite reductase to sulfide. Sulfide is incorporated into cysteine by *O*-acetyl-L- serine (thiol) lyase. Cysteine is used as sulfur donor for the synthesis of methionine and both amino acids are incorporated into proteins. Cysteine is also the precursor for several other sulfur compounds including glutathione [44, 45]. The sulfate uptake by the roots and its transport to the shoots is mediated by specific sulfate transporters [46]. The regulation and expression of sulfate transporters is controlled by the plant's sulfur nutritional status [47]. Sulfate itself or a metabolic product of sulfate assimilation, such as cysteine or glutathione, may be involved in the regulatory control of uptake and transport of sulfate. Despite being highly toxic, the effect of SO_2 on plants is ambiguous, as a part of it is metabolized and utilized by the plants [7, 48–50]. The absorbed SO_2 in the mesophyll cells of the shoot may enter the sulfur reduction pathway either as sulfite or as sulfate. Excess SO_2 is transferred into the vacuole as sulfate, where it is slowly metabolized [51, 52]. Even at relatively low atmospheric concentrations, SO_2 exposure results in an enhancement in the sulfur content of the foliage because of accumulation of sulfate in the vacuole [7, 53]. It is also evident that in addition to sulfate taken up by the roots, plants are able to metabolize sulfur gases, H_2S and SO_2, by the shoot [7, 47, 54]. The gaseous sulfur enters the shoot via open stomata since the cuticle is impermeable to the gas [55]. The rate of uptake depends on the stomatal and mesophyll conductance and the atmospheric concentration. The mesophyll conductance toward SO_2 is very high since SO_2 is highly soluble in the aqueous phase of the mesophyll cells (in either apoplast or cytoplasm). Furthermore, it is rapidly hydrated/dissociated yielding bisulfite and sulfite ions ($SO_2 + H_2O \rightarrow H^+ + HSO^{3-} \rightarrow 2\ H^+ + {SO_3}^{2-}$), which are either reduced in the chloroplast or are enzymatically or nonenzymatically oxidized to sulfate [7, 53]. The stomatal conductance is generally the limiting factor for the foliar uptake of SO_2, which is reflected by a nearly linear relationship between the uptake and the atmospheric SO_2 concentration [53, 56, 57].

E. rudis Endl. plants exposed to 132 and 274 $\mu g\,m^{-3}$ of SO_2 for 8 h per day in open-top chambers for 17 weeks showed no effect on S content at the lower concentrations, but SO_2 at 274 $\mu g\,m^{-3}$ significantly increased the sulfur content of leaves [58]. Appraisal of the effects of the power plant emissions on the nutrient status of six species of tropical trees (two species of evergreen trees *Mangifera indica* and *Eucalyptus* hybrid and four species of deciduous trees *Psidium guajava*, *Cassia siamea*, *Delonix regia*, and *Bougainvillea spectabilis*) from a low rainfall area along a pollution gradient (seasonal average of 49–233 $\mu g\,m^{-3}$ of SO_2) around two coal-fired power plants in India revealed that a higher total foliar sulfur content in all six species at the most exposed location compared to the reference location. Deciduous species showed a greater increase in the foliar sulfur content after the emergence of new leaves possibly due to translocation of sulfur from woody plant parts [59]. Assessment of the sensitivity of *Prosopis ciceraria*, *Azadirachta indica*, and *Phoenix dactilifera* in the vicinity of an oil refinery based on sulfate accumulation showed that plants responded differently to SO_2 exposure. Plants grown in the close vicinity have maximum sulfate accumulation and injury than the distant ones [60]. Chinese cabbage is highly susceptible to sulfur dioxide showing a linear relation between the rate of uptake of SO_2 and the atmospheric concentrations (0.03–1.4 $\mu l\,l^{-1}$). Biomass of cabbage was reduced upon prolonged exposure to $\geq$0.1 $\mu l\,l^{-1}$ of SO_2 and resulted in an increase in SO_4^{2-}, water-soluble nonprotein thiols, and total S content of the shoot at concentrations $\geq$0.1 $\mu l\,l^{-1}$; however, the ratio of organic S to total S and organic N content was not affected. The impact of SO_2 on Chinese cabbage seemed to be ambiguous; SO_2 taken up by the shoot also served as a source of S for growth and was even beneficial when the SO_4^{2-} supply to the root was cut off. A 5 day exposure of plants to 0.06–0.18 $\mu l\ SO_2\ l^{-1}$ resulted in an alleviation of the development of S deficiency symptoms upon SO_4^{2-} deprivation. An atmospheric SO_2 level as low as 0.06 $\mu l\,l^{-1}$ appeared to be sufficient to cover the plants' S requirement for growth. The N/S ratio of shoot and root was much lower in SO_4^{2-} sufficient plants than in SO_4^{2-}-deprived plants. Exposure of SO_4^{2-}-deprived plants to SO_2 resulted in a decrease in the N/S ratio of the shoot, but did not affect that of the root. The N/S ratio of the shoot decreased with increasing SO_2 levels as a consequence of the increase in total S and SO_4^{2-} content. In contrary, the N/S ratio of shoot and root of SO_4^{2-}-sufficient plants was not significantly affected upon exposure to 0.06–0.18 $\mu l\,l^{-1}$ of SO_2. SO_4^{2-} deprivation resulted in a shift in shoot-to-root biomass partitioning during growth in favor of root production, which was not rapidly alleviated when SO_2 was used as S source for growth [61]. According to Dwivedi *et al.* [62], there is a positive correlation between ambient sulfur dioxide and sulfate (accumulation) in the leaves.

SO_2 readily reacts with water and forms sulfite ions that impact deleteriously on plant health. Modulation of the level of sulfite oxidase (SO) that catalyzes the transformation of sulfites to the nontoxic sulfate showed that *Arabidopsis* and tomato plants can be rendered resistant or susceptible to SO_2/sulfite. Plants in which sulfite oxidase expression was abrogated by RNA interference (RNAi) accumulated relatively less sulfate after SO_2 application and showed enhanced induction of senescence- and wounding-associated transcripts, leaf necrosis, and chlorophyll

bleaching. In contrast, SO overexpression lines accumulated relatively more sulfate and showed little or no necrosis after SO_2 application. The transcript of sulfite reductase, a chloroplast-localized enzyme that reduces sulfites to sulfides, was shown to be rapidly induced by SO_2 in a sulfite oxidase-dependent manner. Transcripts of other sulfite-requiring enzymatic activities such as mercaptopyruvate sulfur transferases and UDP-sulfoquinovose synthase 1 were induced later and to a lesser extent, whereas SO was constitutively expressed and was not significantly induced by SO_2. The results imply that plants can utilize sulfite oxidase in a sulfite oxidative pathway to cope with sulfite overflow [63].

6.3.3 Photosynthesis

There have been numerous efforts to measure the effect of SO_2 on metabolic processes in plants that can affect photosynthesis and other related processes such as stomatal conductance, photochemical efficiency, carbon dioxide assimilation, chlorophyll content, dark respiration, and carbohydrate metabolism. In a study designed to examine the changes in leaf gas exchange resulting from SO_2 exposure, Gerini *et al.* [64] exposed maize (*Zea mays* L.) in fumigation chambers to 113, 186, or 291 $\mu g\,m^{-3}$ of SO_2 for 4 weeks resulting in a 20% decrease in photosynthetic activity in plants exposed to 113 and 186 $\mu g\,m^{-3}$ of SO_2. They observed photosynthetic activity decreases by 10% at 291 $\mu g\,m^{-3}$ of SO_2 compared to control plants. Stomatal conductance, transpiration rate, and intercellular/ambient CO_2 were enhanced at the lowest SO_2 treatment, but decreased to near control levels at 291 $\mu g\,m^{-3}$ of SO_2 treatment. In contrast, water use efficiency and CO_2 assimilation rate declined at the lowest concentration of SO_2 and then increased at the higher SO_2 levels but not back to control levels. Sulfur dioxide levels used were representative of ambient SO_2 levels observed in the environment. The authors attributed the decrease in photosynthetic activity to reduced mesophyll assimilation capacity. Stomatal effects were ruled out as stomatal conductance and intercellular CO_2 were enhanced at these levels of SO_2. Utilizing an open-air fumigation system, Darrall [65] examined the effects of SO_2 at ambient, low (100 μg), medium (113 μg), and high levels (126 μg) on photosynthesis, dark respiration, transpiration, stomatal conductance, and internal CO_2 concentration and correlated the changes with grain yield in winter barley, *Hordeum vulgare* cv. Igri. Experiments were conducted for 3 years and the SO_2 concentrations varied within each year. The average concentrations for the highest SO_2 treatment for each year were 100, 113, and 126 $\mu g\,m^{-3}$. It was observed that SO_2 significantly increases net photosynthesis on some occasions, significant decreases were also frequently observed, and most of the photosynthetic changes were transient and were attributed to simultaneous changes in stomatal conductance and transpiration. Dark respiration was significantly enhanced at 84 and 113 $\mu g\,m^{-3}$ of SO_2. The author concluded that increase in dark respiration could have been resulted from the enhanced detoxification and repairing processes.

Panigrahi *et al.* [66] determined the effect of SO_2 on chlorophyll content, by exposing 20, 40, 60, 80, and 100 days old rice (*Oryza sativa* L.) and 15, 30, 45, and 50

days old mung bean (*Phaseolus aureus* R.) cv. Dhauli to 655–5240 $\mu g\,m^{-3}$ of SO_2 for 6–48 h. Chlorophyll content in rice and mung bean decreased by 20 and 40% at 655 and 1310 $\mu g\,m^{-3}$ of SO_2, respectively. The decrease was directly related to the exposure period. Exposure to 5240 $\mu g\,m^{-3}$ of SO_2 level resulted in almost complete destruction of chlorophyll. It was summarized that a decrease in chlorophyll leads to a decrease in growth parameters including biomass, productivity, and yield. The gas exchange response of 2-year-old seedlings of oak (*Quercus pubescens* Wild.) and Turkey oak (*Q. cerris* L.) on exposure to 73, 160, and 244 $\mu g\,m^{-3}$ of SO_2 for 23 weeks in fumigation chambers was evaluated by Lorenzini *et al.* [67]. After 11 weeks of exposure, a significant decrease in photosynthetic activity, stomatal conductance, transpiration rate, and water use efficiency was noticed. In addition, the vapor pressure deficit increased with increasing SO_2 concentration, but the internal/ambient CO_2 ratio was not affected. For *Q. cerris* there was a significant linear decrease in photosynthetic activity, vapor pressure deficit, and water use efficiency, but stomatal conductance and transpiration rates remained unaltered. The internal/ambient CO_2 ratio increased by 15% at 244 $\mu g\,m^{-3}$ of SO_2. Ranieri *et al.* [68] investigated long-term exposure of barley cv. Panda and Express to 210 $\mu g\,m^{-3}$ of SO_2, in a greenhouse, to establish whether negative impacts of SO_2 could be linked to specific changes in the photosynthetic apparatus. Exposure for 75 days did not result in any visible injury to either cultivar, while photosynthetic activity decreased by 29 and 49% in cultivars Panda and Express, respectively. Stomatal conductance reduced by 56% (Panda) and 58% (Express), and the whole electron transport chain activity was reduced by 27 (Panda) and 29% (Express). There was 7 and 11% and 18 and 24% reduction in electron transport activities of photosystem I and II in cv. Panda and Express, respectively. Chlorophyll a decreased by 44 (cv. Panda) and 10% (cv. Express), while the corresponding decrease in carotenoids was 46 and 10%. Pigment–protein complexes from thylakoid membranes did not show any qualitative or quantitative differences between control and SO_2-exposed plants. The effect of SO_2 treatments (1.3 and 0.6 ppm) on photosynthesis of *Augea capensis* Thumb, a succulent exhibiting C3 mode of photosynthesis, was investigated on the basis of CO_2 assimilation and chlorophyll a florescence measurements. The study revealed that the inhibitory effects on photosynthesis were induced only when SO_2 fumigation occurred in the dark. An inhibition of 38 and 62% in carboxylation efficiencies and CO_2 saturated rates, respectively, in photosynthesis was observed. However, these effects occurred only at the highest concentration and were fully reversible, indicating no permanent metabolic damage. Only minor effects on photosystem II were observed, indicating that photochemical reactions were not the primary site of inhibition. Cellular capacity for SO_2 detoxification differs during day and night [69].

6.3.4
Stomatal Conductance and Transpiration

Any alteration in the gaseous composition of the atmosphere affects terrestrial plants. In most cases, stomata are affected worst by the environmental pollution. Evolution has provided highly complex mechanisms by which stomata respond to a wide range

of environmental factors to balance the conflicting priorities of carbon gain for photosynthesis and water conservation. These mechanisms involve direct responses of the guard cells to the aspects of the aerial environment and hormonal communication within the plant enabling conductance to be adjusted according to soil moisture status. Various aspects of these delicately balanced mechanisms can be disturbed by air pollutants. Stomata are the main avenues for the diffusion of gases and water vapor in plants. Any factor that influences stomatal conductance is likely to affect plant–water relations as well as diffusion of carbon dioxide and oxygen. Sulfur dioxide exerts a marked influence on the stomatal conductance [70]. Sulfur dioxide-induced stomatal opening has great physiological and ecological implications. It seems certain that maximum damage to plants by SO_2 occurs when the stomata are open. Prolonged opening of stomata results in excessive loss of water through transpiration. Consequently, the water requirement of plants in the polluted areas will be relatively greater. Any condition that promotes stomatal opening will enhance sulfur dioxide diffusion and damage, whereas any factor that can nullify SO_2-induced stomatal opening may provide protection against the gas injury. High humidity and fog increase the sensitivity of plants to the gas and promote the formation of acidic mist. Under such conditions, stomata remain open for longer periods, permitting greater diffusion of sulfur dioxide and other air pollutants into the leaf [4]. Khan and Khan [1] reported that exposure to SO_2 decreased the number and size of stomata but increased the number and length of trichomes on both leaf surfaces. Stomatal aperture was significantly wider in plants exposed to 286 or 571 $\mu g\, m^{-3}$ of SO_2. Stomatal aperture was directly related to foliar injury and reductions in growth, yield, and leaf pigments. Number and size of stomata in the leaves of eggplants grown at sites 1 and 2 km away from the SO_2 source (thermal power plant) were decreased, but their apertures were wider. Number and length of trichomes were greater at the polluted sites, being more on upper leaf surface [71].

Han [72] investigated the relationship between stomatal infiltration and SO_2 injury and the protective effect of abscisic acid (ABA). The study revealed that the effect on infiltration of the same species under different SO_2 concentration was little less than one grade, while K^+ efflux increased with the increase in SO_2 amount absorbed by the leaves. Higher ABA solution concentration and the K^+ efflux were lower when the leaves were sprayed with ABA solution. When leaves sprayed with ABA solution were smoked with 2.5 $mol\, l^{-1}$ for 4 h, the infiltration of leaves with 30 $mol\, l^{-1}$ ABA solution dropped by 1.5–3.0 and K^+ concentration on leaves decreased by 36.5%–54.8%. It indicates that the ABA solution on leaves has a remarkable effect of protection of SO_2 injury. Dhir *et al.* [73] investigated the stomatal responses of *Cichorium intybus* leaves to sulfur dioxide treatment at different stages of plant development in 50 day-old *C. intybus* L. plants exposed to 1 ppm sulfur dioxide gas 2 h per day for 7 consecutive days that resulted in a greater length and width of stomatal apertures on lower and upper epidermis. Stomata were longer on the adaxial epidermis, but shorter on the abaxial epidermis, except at the preflowering stage; moreover, stomatal widths varied widely. Compared to the controls, the abaxial epidermis on treated leaves showed consistently lower stomatal densities and stomatal indices. This was also true for the adaxial epidermis during the postflower-

ing stage. The stomatal conductance was reduced in the SO_2-exposed plants, but intercellular CO_2 concentrations increased at the preflowering stage and, subsequently, declined. According to Rao and Dubey [74] stomatal conductance decreased by 26–28% in *Zizyphus mauritiana* Lam., *Syzygium cuminii* L., *A. indica* A. Juss, and *M. indica* L. at the sites contaminated with of 90 $\mu g\,m^{-3}$ of SO_2 in comparison to the control site. Wali *et al.* [75] found that stomatal conductance and intercellular CO_2 concentration in *C. officinalis* L. decreased with 0.5 ppm SO_2 treatment, the reverse being the case with higher concentration, that is, 1.0 and 2.0 ppm.

6.3.5 Leaf Pigments

The adverse effects of sulfur dioxide on photosynthesis are partly due to its action on photosynthetic pigments. Sulfur dioxide can react with chlorophyll molecules in three distinct ways: bleaching (i.e., loss of color), phaeophytinization (i.e., degradation of chlorophyll molecules to photosynthetically inactive pigment phaeophytin), and the process responsible for a blueshift in the pigment spectrum as observed in lichens [76]. Prasad and Rao [77] studied the relative sensitivity of soybean (*Glycine max* (L.) Merr.) and wheat (*Triticum aestivum* L.) to SO_2 and found that the amount of total chlorophyll in SO_2-treated wheat plants increased at low SO_2 doses, but this was not the case with soybean plants. However, at 120 and 160 ppm h^{-1} of SO_2, total chlorophyll content reduced by 19% in soybean and 17% in wheat. The loss of chlorophyll a was relatively greater than that of chlorophyll b in both the species following exposure. Chlorophyll a and b in treated soybean plants were reduced, respectively, by 21 and 12% at the cumulative dose of 120 ppm h^{-1} of SO_2 and in wheat by 19 and 14% at 160 ppm h^{-1} of SO_2. The maximum reductions in the amounts of carotenoids, that is, 12 and 7% were recorded in soybean and wheat plants at the cumulative doses of 120 and 160 ppm h^{-1} of SO_2, respectively. Dhir *et al.* [73] reported that photosynthesis rate was reduced in the SO_2-exposed plants, but intercellular CO_2 concentrations increased at the preflowering stage and declined subsequently. Chlorophyll a, carotenoid, and total chlorophyll contents increased at the preflowering stage and then decreased. The level of chlorophyll b was reduced throughout plant development compared to the untreated controls. Prakash *et al.* [78] investigated the effect of three different concentrations of sulfur dioxide (320, 667, and 1334 $\mu g\,m^{-3}$) exposure on the chlorophyll contents in *Raphanus sativus* L. and *Brassica rapa* L. and found that both the chlorophyll a and b content decreased with increasing concentration, maximum decrease being at the highest concentration, that is, 1334 $\mu g\,m^{-3}$ of SO_2. However, chlorophyll a showed more reduction than chlorophyll b. Wang *et al.* [79] reported the effects of artificial acid rain and SO_2 on characteristics of delayed light emission (DLE) by using a home-made weak luminescence detection system with the lamina of zijinghua (*Bauhinia variegata*) and soybean (*G. max*) as testing models. The results showed that the changes in DLE intensity of green plants reflect the changes in chloroplast intactness and function. It has been concluded that DLE may provide an alternative means for evaluating environmental acid stress on plants. Seedlings of maize cv. CO-1 when exposed to

SO_2 at LD_{50} underwent a significant decline in total chlorophyll, chlorophyll a and b contents, and carotenoids [80]. Wali *et al*. [75] evaluated the anatomical and functional responses of *C. officinalis* to 0.5, 1.0, and 2.0 ppm SO_2 and found that the high SO_2 doses caused significant decline in the photosynthetic pigments at each stage of plant development, although 0.5 ppm concentration had a stimulatory effect on leaf pigmentation.

6.3.6 Growth and Yield

The physiological and/or biochemical disorders induced by SO_2 are finally manifested as the structural and quantitative alterations in plants. Since plants show varied response to SO_2 under varied exposure conditions, the effects under ambient and simulated conditions are different and being presented separately.

6.3.6.1 Ambient Condition

Since the first observation of a plant disease incited by an air pollutant under ambient conditions [81], many such studies have come up, but a general concern among scientists toward suppressive effects of pollutants on plant growth emerged in the 1940s. The smog injury on plant foliage in Los Angeles provided an impetus to research on phytotoxic effects of pollutants [82]. The forest decline, in fact, drew the attention of environmentalists and researchers on the response of forest trees to sulfur dioxide. Haywood [83, 84] reported severe damage to different pine and oak species from smoke (primarily SO_2) of a copper smelter in California. Sulfur dioxide from a copper smelter in British Columbia also caused severe damage to ponderosa pine (*P. ponderosa* Douglas), lodgeople pine (*P. contorta* Douglas), Douglas fir (*Pseudotsuga menziesii* Carriere), western larch (*Larix occidentalis* Nutt), and so on up to 52 mile southward [85]. Ponderosa pine and Douglas fir grown over 5200 acres in Montana, USA, has shown a gradual decline. Needle necrosis and premature senescence are the most peculiar symptoms of sulfur dioxide damage [86]. Sulfur dioxide from copper smelters [87], nickel smelters [88], iron sintering plants [89], and coal-fired thermal power plants [90, 91] has significantly contributed to the decline of forest trees in the United States and Europe.

SO_2 in the coal smoke may cause chlorosis and browning of leaves, suppress plant vigor, inhibit fruit setting, and decrease the yield as observed in trees such as *Dalbergia* and *Psidium* [92, 93], weeds such as *Commelina benghalensis*, *Croton bonplandianum*, and *Euphorbia hirta* [94–96], grasses such as *Cynodon dactylon*, *C. dactylis glomerata*, and *Lolium perenne* [97, 98], cereals such as wheat and barley [99, 100], and vegetables such as tomato, okra, eggplant, and cucurbits [22, 23, 96, 101, 102]. Garcia *et al*. [103] studied the response of two populations of holm oak (*Q. rotundifolia* Lam.) to SO_2. One-month-old potted plants were grown for 130 days in an atmosphere enriched with SO_2 (0.23 ppm, 14 h per day) in a growth chamber. Both northern and southern plants underwent a significant decrease in growth rate. The southern population was more sensitive to the treatment, as reflected in the bigger decrease in both growth and photosynthesis rates. The author concluded that the

differences in resistance appear to be related to the biogeographic origin of the populations underlining the importance of biogeographic aspects in studies of resistance to air pollutants. Abdul-Wahab and Yaghi [60] made an assessment of the impacts of long-term SO_2 emissions from an oil refinery on three different plant species, namely, *P. cineraria*, *A. indica*, and *P. dactilifera* using sulfate contents of the plants as bioindicators for monitoring SO_2 concentration. The results showed that the three plant species responded differently to SO_2 in terms of sulfate contents. All three species were found to be sensitive to SO_2 exposure and the concentration of sulfate was found to be much higher in plants closer to the refinery.

6.3.6.2 **Simulated Condition**

There are numerous experiments/studies that have been conducted in simulated conditions for the evaluation of the effect of SO_2 on plant growth and yield. Coleman *et al.* [104] presented results on the variability of biomass production for wild radish (*R. sativus* x *raphanistrum*) and cultivated radish (*R. sativus*) cv. Cherry Belle exposed to 262, 629, or 1048 $\mu g\,m^{-3}$ of SO_2 in fumigation chambers with 10 h light period for 24, 30, or 35 days. Variability in biomass production increased with increase in SO_2 exposure period on radish. It has been concluded that genetic differences between the individual plants (differential sensitivity to SO_2) might be the reason for the increased variability as the SO_2 concentration increased. Weigel *et al.* [105] investigated growth and yield responses of different crop species to long-term fumigation with SO_2 in open-top chambers. Potted plants of commercial cultivars of rape (*B. napus* L., cv. callypso), summer barley (*H. vulgare* L., cv. Arena and Hockey), and bush beans (*P. vulgaris*, cv. Rintintin and Rosisty) were continuously exposed in open-top chambers to SO_2 for the whole growing season. Treatments consisting of charcoal-filtered air (CF) and CF supplemented with four levels of SO_2 resulted in mean exposure concentrations of approximately 8, 50, 90, 140, and 190 $\mu g\,m^{-3}$. With the exception of the 1000 seeds weight, which was slightly reduced, dry matter production and yield parameters of rape remained unaffected by all SO_2 concentrations or were even stimulated. Compared to CF, vegetative growth of both bean cultivars was reduced by 10–26% at all SO_2 levels, with significant effects only for cv. Rintintin, however. While all SO_2 additions reduced significantly the yield (dry weight of pods) of the bean cv. Rosisty by 17–32%. The cv. Rintintin showed a significant reduction up to 42% only at the two highest pollutant concentrations. Dry matter production of the barley cultivars was mainly impaired at SO_2 concentrations $>100\,\mu g\,m^{-3}$ with a reduction of 30–52%. While nearly all yield parameters of cv. Hockey reacted similar to the dry matter production, the yield of cv. Arena was reduced already at the low SO_2 levels. At a treatment concentration of 90 $\mu g\,m^{-3}$ of SO_2, a significant yield loss of 30% was recorded. A reduction of the 1000 grains weight mainly contributed to these yield losses observed for both barley cultivars. It has been concluded that SO_2 concentrations within the range of 50–90 $\mu g\,m^{-3}$ are potentially phytotoxic to some crop species.

Murray and Wilson [106] conducted an open-top chamber experiment to examine the effect of sulfur dioxide exposure on sulfur accumulation and alteration of growth and yield of barley (*H. vulgare* L.) cv. Schooner. Exposure to 110 $\mu g\,m^{-3}$ of SO_2 for 4 h

per day for 79 days increased the shoot length and weight by 10%. This increase in growth was attributed to the fertilizer effect of SO_2. SO_2 at 317 $\mu g\, m^{-3}$ or higher significantly decreased the height, weight, number of tillers, and yield in barley. These increases were proportional to the concentration of the SO_2 exposure. In addition, the shoot sulfur content increased linearly from 0.14% (control plants) to 0.77% at 1354 $\mu g\, m^{-3}$ of SO_2. Effects of long-term SO_2 (132 and 274 $\mu g\, m^{-3}$ for 8 h per day for 17 weeks) exposure on growth and development of *Eucalyptus rudis* Endl. plants in open-top chambers was studied by Clarke and Murray [58]. Plants exposed to 132 $\mu g\, m^{-3}$ of SO_2 for 17 weeks increased the height, leaf area, and dry weight of leaves because of an increase in size of leaves, but total number of leaves remained unaltered. Sulfur dioxide levels of 274 $\mu g\, m^{-3}$ did not affect plant height, leaf area, and dry weight of leaves, but increased the rate of leaf abscission. Kropff [107] performed field experiments with an open-air fumigation system to interpret and explain the observed yield loss in broad bean (*Vicia faba* L.) by quantifying the contribution of different physiological processes. Fumigation with 74 $\mu g\, m^{-3}$ of SO_2 throughout the growing season resulted in 9 and 10% decrease in dry matter and pod yield, respectively. These losses were accompanied by visible injury (brown/red spots), which progressed from the oldest leaves upward and also resulted in some leaf abscission. When exposed to 165 μg of SO_2, dry matter and yield were reduced by 17 and 23%, respectively. The dry matter production was primarily decreased due to loss of green leaf area in SO_2 exposed plants. In an open-top chamber study, barrel medic (*Medicago truncatula* Gaerm.) cv. Paraggio was exposed to 107–1349 $\mu g\, m^{-3}$ of SO_2 for 4 h per day, 7 days per week for 72 days [108]. Less than 10% reduction in the plant growth was recorded at concentrations upto 314 μg SO_2; however, at 668 $\mu g\, m^{-3}$ of SO_2, there was 40–50% reduction in growth accompanied by 85% increase in the S concentration. There was significant reduction in flowering with the increase in SO_2 concentration, and at 1349 $\mu g\, m^{-3}$ of SO_2, there was little or no plant growth. Potato (*Solanum tuberosum* L.) was exposed to 288 or 786 $\mu g\, m^{-3}$ of SO_2, for 105 days in closed top field chambers for 4 h per day under well-watered or water-stressed conditions to study the interactive effects of soil water stress and SO_2 [109]. Visible symptoms appeared after 9 weeks of exposure to 288 $\mu g\, m^{-3}$ of SO_2 and after 6 weeks of exposure to 786 $\mu g\, m^{-3}$ of SO_2. At harvest, the leaf S content of well-watered plants had increased by more than 100 and 125% in the 288 and 786 $\mu g\, m^{-3}$ SO_2 treatments, respectively. When water stressed, the lower SO_2 treatment had little effect on S content, whereas the 786 $\mu g\, m^{-3}$ SO_2 treatments resulted in a 100% increase in leaf sulfur. Leaf chlorophyll of 35 day-old leaves from well-watered plants decreased significantly, by approximately 30% at 288 $\mu g\, m^{-3}$ of SO_2 and by 40% at 786 $\mu g\, m^{-3}$ of SO_2. In contrast, water stress resulted in a maximum chlorophyll loss of 11% at 786 $\mu g\, m^{-3}$ SO_2. Exposure of well-watered potato plants to 786 $\mu g\, m^{-3}$ of SO_2 resulted in a significant decrease in dry weight of leaves (25%) and tuber (35%) compared to control. In contrast, dry weight reductions in water-stressed plants did not occur on exposure to SO_2. This might be due to increased stomatal resistance in response to mild water stress that limits SO_2 uptake.

Colls *et al*. [110] used an open-air fumigation system to expose winter barley (*H. vulgare* L.) cv. Igri to a single dose (defined as concentration × time) of SO_2 to

determine if concentration peaks or long-term averages had the greatest effects on the plants. The treatments were based upon achieving an equivalent dose of 534 μg m^{-3} of SO_2 for 6 days. The treatments included continuous exposure to 89 μg m^{-3} of SO_2 for 6 days, 178 μg m^{-3} of SO_2 for 3 days, followed by ambient air for 3 days, or 534 μg m^{-3} of SO_2 for 1 day followed by 5 days of exposure to ambient air. This 6-day cycle was repeated 24 times during the growing season. There were no effects on shoot dry weight accumulation or on grain yield in any treatment. This was attributed to plants' ability to metabolize excess sulfate during the SO_2-free days. Julkunen-Tiitto *et al.* [111] studied the effects of SO_2 exposure on growth and on phenol and sugar production in six clones of willow (*Salix mysrsinifolia* Salisb). A disruption in secondary metabolism could alter plant response to herbivores and microorganisms. Clones were exposed to 300 μg m^{-3} of SO_2 for 7 h per day, 5 days per week, for 3 weeks in fumigation chambers. Salicin and chlorogenic acid content decreased by 15 to >70% depending on clone, while there was no significant effect on salicortin, 2′-*O*-acetylsalicortin, (+)-catechin, and two unknown phenolics. Since SO_2 exposure did not affect salicortin and 2′-*O*-acetylsalicortin (key molecules in the defense chemistry), it was concluded that willow resistance to herbivory and microorganism attack was not reduced. Glucose, fructose, and sucrose contents were not significantly affected. Willow exposed to 300 μg m^{-3} of SO_2 produced 14–48% greater biomass (leaf, stem, and root dry weights) compared to control plants. Exposure to SO_2 at 0.1 and 0.2 ppm in *M. javanica* individually caused significant reduction in plant growth of pea; moreover, this reduction was much greater in joint treatment [112].

Greenhouse studies were conducted by Ashenden *et al.* [113] to examine the effect of SO_2 on growth of 41 British herbaceous species to determine whether the species differed in their sensitivity to SO_2. Plants were exposed to a constant background concentration of 262 μg m^{-3} of SO_2 with peaks applied during daylight. During the first 4 weeks, peak SO_2 concentration used was 524 μg m^{-3} for 2 h, twice a week. For the next 3 weeks, 786 μg m^{-3} was applied for 3 h, thrice a week. Finally, for the last 3 weeks, peaks of 786 μg m^{-3} were applied for 3 h, five times a week to maximize any growth differences between the tested species. There was 43% reduction in total dry matter content of different plants. The mean response of all 41 species was a 25% decrease in total dry mass. Of the seven statistically significant responses of total leaf area, there was an average decrease of 40%. The mean response for all 41 species was a decrease of 10% in total leaf area. Leaf area ratio increased by 45% in 20 plants, and an average increase of 23% was recorded for all 41 species. In 13 species, an average decreases of 36% in the root:shoot ratio occurred due to SO_2 exposure, whereas for all species the decrease was 14%. This study reveals that while there were differences in growth response of the species tested and the same responses may not be observed under field conditions because SO_2 concentrations in the field are not expected to be as high as those used in this study. Plants growing in natural communities may also respond differently from plants grown in individual containers. Moreover, the nutrient supply in this study was nonlimiting, while in the field nutrients may be limiting and may alter responses to SO_2. Agrawal and Verma [10] determined whether varying the levels of nitrogen (N), potassium (K), and phosphorous (P) in the growth medium could affect the response of wheat (*T. aestivum* L.) cv. Malviya 206 and Malviya

234 to SO_2. Thirty-day-old plants were exposed to $390 + 20\,\mu g\,m^{-3}$ of SO_2 for 4 h per day, 5 days a week, for 8 weeks in open-top chambers. Visible injury symptoms appeared earlier and were the greatest in both cultivars grown with no additional nutrients. Unfertilized plants exposed to SO_2 had the greatest dry weight, height, and yield reductions, while plants grown with recommended or two times the recommended levels of NPK were able to alleviate SO_2 effects to the greatest extent. Leaf area and total chlorophyll content decreased significantly when plants were exposed to SO_2. Ascorbic acid was significantly reduced in treated plants as it was utilized in removal of free radicals generated by SO_2 in foliar tissue. Sulfur dioxide treatment also resulted in an increase in sugars and a decrease in starch. Sulfate-sulfur increased in treated plants and the greatest increase was in plants grown with no additional nutrients. An increase in the root:shoot ratio was also observed in SO_2-treated plants suggesting a modification in the carbon allocation pattern when plants were exposed to SO_2. It was concluded that both nutrient deficiency and SO_2 reduced the considered parameters, but addition of NPK in different combinations ameliorated the adverse effects of SO_2. Dhir *et al.* [73] observed that when 50 day-old *C. intybus* L. plants were exposed to 1 ppm sulfur dioxide gas, 2 h per day for 7 consecutive days, the number, dimensions, area, and biomass of leaves were less in the treated plants. Growth dynamics of wheat, *T. aestivum* L., cv. Aurelio, Mec Manital, and Chiaram, was investigated in relation to SO_2 exposures by Lorenzini *et al.* [99]. All the cultivars responded differently to long-term exposure to SO_2. The cv. Mec showed significant reductions in several of the growth and yield parameters, while the other cultivars were only marginally affected. Fumigation with SO_2 reduced the yield of cv. Mec by 33%. Two-week-old wheat cv. Banks seedlings exposed to 0.004, 0.042, 0.121, 0.256, or $0.517\,\mu l\,l^{-1}$ of SO_2 for 4 h per day for 79 days resulted in a significant reduction in plant height, shoot weight, development stage, number of tillers, ear weight per plant, average ear weight, and total number of ears at and above $0.042\,\mu l\,l^{-1}$ [48]. SO_2 at LD_{50} could not affect growth of maize (*Z. mays*) cv. Co-1 though the shoot length decreased [80]. Tiwari *et al.* [114] studied the seasonal variations and effects of ambient air pollutants on the root, shoot length, number of leaves per plant, leaf area, and root and shoot biomass of lettuce, *Beta vulgaris* L. cv. Allgreen, at a suburban site situated in dry tropical area of India, experiencing elevated levels of ambient air pollutants. Air monitoring data showed that mean concentrations of SO_2 and NO_2 were higher during winter. Plants grown in nonfiltered chambers showed stunted growth, reductions in biomass and yield, and modification in biomass allocation pattern compared to those grown in charcoal-filtered air. Biomass allocation pattern revealed that during summer photosynthate allocation to roots reduced with consequent increment in leaf weight ratio, which helped in sustaining nutritional quality of the lettuce even after more yield reductions in NFCs compared to FCs.

6.3.7
Pollen and Fertilization

Pollution also influences reproductive processes in terms of smaller pollen sizes, reduced germination rates, and shorter pollen tubes. Reduced seed size, seed

germination capacity, and a lower share of flowering trees in polluted areas have been reported [115, 116]. Effects of wet and dry exposure, both *in vivo* (on the anthers) and *in vitro* (culture dishes) on germination of pollen from oilseed rape (*B. napus* L.) cv. Tapidor and Libravo was investigated by Bosac *et al.* [117]. For *in vivo* treatments, inflorescences were exposed in special chambers (excluding the rest of the plant) to 524 $\mu g\,m^{-3}$ of SO_2 for 6 h. *In vitro* exposures (wet or dry) lasted for 3 h. Exposure to 524 $\mu g\,m^{-3}$ of SO_2 had no effect on germination or pollen tube length, *in vivo* or *in vitro* (dry); however, there was a significant reduction in germination when pollens were exposed to SO_2 while in unbuffered medium droplets. Pollen tube length was also greatly reduced under these conditions, but too few pollen grains germinated and grew to calculate reliable mean values. It was concluded that the reduction in germination in the unbuffered medium was due to acidification of the medium (pH dropped form 6.5 to 5.5) during SO_2 exposure. Sulfur dioxide is highly soluble in water; therefore, it would get dissolved and acidify the medium during exposures. Agrawal *et al.* [118] utilized a nightshade (*S. nigrum*) complex, which exhibits three natural cytotypes (diploid *S. americanum*, tetraploid *S. villosum*, and hexaploid *S. nigrum*) to determine the effects of SO_2 on pollen chromosomes. Flowering plants were exposed to 524 $\mu g\,m^{-3}$ of SO_2 for 2 h pre day for 3, 7, or 11 days. When pollen mother cells (PMCs) were examined, it was found that meiotic chromosomal abnormalities were highest in diploid plants (19.67–26.0%) and least in hexaploid plants (4.45–7.0%). In addition, abnormalities increased with length of exposure for all plants. Pollen sterility followed the same pattern as chromosomal abnormalities, 19.5–21.6% in diploid, 13–15% in tetraploid, and 10–13% in hexaploid, with sterility increasing with length of exposure. The authors concluded that the observed abnormalities might have resulted either from free radical splitting of phosphodiester linkages of DNA or from bisulfite combining with cytosine or uracil that may result in alteration of DNA or RNA functions.

6.3.8
Proteins and Antioxidant Enzymes

Several studies have been conducted to assess the effects of SO_2 on plant proteins and antioxidant enzymes and to explain the role of antioxidant enzymes in SO_2 tolerance and its possible mechanism(s). The role of superoxide dismutase (SOD) in defense against SO_2 toxicity was investigated using leaves of poplar and spinach by Tanaka *et al.* [119]. Young poplar leaves with five times the SOD of the old leaves were more resistant to the toxicity of SO_2. The SOD activity in poplar leaves was increased by fumigation with 0.1 ppm SO_2, and this was more evident in young leaves than in the old ones. The poplar leaves having high SOD activity due to 0.1 ppm SO_2 fumigation were more resistant to 2.0 ppm SO_2 than the control leaves. The finding suggested that SO_2 toxicity is in part due to the superoxide radical and that SOD participates in the defense mechanism against SO_2 toxicity. Elemental sulfur and many sulfur-containing compounds such as cysteine-rich antifungal proteins, glucosinolate (GSL) and phytoalexins, play important role in plant disease resistance. Pierre and Queiroz [120] investigated the enzymatic and metabolic changes in bean (*P. vulgaris* L.) leaves on

continuous exposure to subnecrotic levels of SO_2. The study revealed a rapid increase in enzyme capacity at 0.1 ppm or 3 $\mu g\, m^{-3}$ of SO_2. Peroxidases are the key enzymes of the metabolic pathways. The composition of organic acids, amino acids, and polyamines was altered with change in enzymes. The effect of low levels of SO_2 (3, 5, and 10 ppm) on peroxidase was evaluated on the foliage of *B. nigra* L., *P. radiatus* L. (SO_2 sensitive), and *Z. mays* L. (SO_2 resistant) [121]. SO_2 enhanced the peroxidase activity in all species, but least increase was observed in *Z. mays*. Six weeks fumigation with 3, 5, and 10 ppm SO_2 increased peroxidase activity by 32, 40, and 45% in *B. nigra*; by 25, 30, and 43% in *P. radiatus*; and by 4, 8, and 13% in *Z. mays*, respectively. Peroxidase activity was found to increase as a function of concentration and duration of SO_2 exposure. SO_2 fumigation increased the intensity and thickness of individual isoenzyme bands without affecting the overall isoenzyme pattern except in *P. radiatus*, where a 6-week exposure altered the isoenzyme pattern. The sulfite turnover rate was faster in *Z. mays* compared to *B. nigra* and *P. radiatus*. It was postulated that the high peroxidase activity and high sulfite turnover rate possessed by *Z. mays* provides a relatively high resistance against SO_2 toxicity.

The amount of glutathione (GSH), an important element in both plant and insect antioxidant systems, is known to increase after exposure to stresses. The effect of SO_2 on GSH concentration in soybean was investigated [122]. GSH levels were found to vary with SO_2 concentration in the same manner as did the insect response. Chauhan [123] performed a study on the early diagnosis of SO_2 stress and the mechanism of SO_2 damage in crop plants by measuring volatile emissions from treated tissues. Emissions from tomato (*Lycopersicon esculentum* Mill.), mung bean (*Vigna radiata* L.), and maize (*Z. mays* L.) were measured after exposure to 262 $\mu g\, m^{-3}$ of SO_2 for 2 h per day or 524 $\mu g\, m^{-3}$ of SO_2 for 1 h per day. Tomato and maize were exposed for 60 days and mung bean was exposed for 45 days. Ethylene, ethane, acetaldehyde, and ethanol contents were measured at 15 day intervals. Ethylene emissions substantially increased in all the three species, until visible injury symptoms (chlorosis followed by necrosis) appeared, after which ethylene concentration declined. Ethane emissions were detected just prior to the appearance of visible injury symptoms and increased with increase in injury levels. It was suggested that ethane production was a result of lipid peroxidation caused by sulfate oxidation. To verify this, an additional experiment with mung bean was performed to establish the relationship between antioxidants and SO_2 damage. Addition of antioxidants substantially reduced ethylene and ethane production supporting the idea that lipid peroxidation was caused by free radicals resulting from sulfite oxidation. Acetaldehyde and ethanol emissions increased as exposure duration increased up to 45 days, but emissions declined after the appearance of visible injury symptoms. As acetaldehyde and ethanol are not normal by-products of aerobic metabolism, it was concluded that their production was a result of SO_2-induced alteration of respiratory metabolism. The rates of emissions of ethane, acetaldehyde, and ethanol were related to the degree of SO_2 resistance displayed by the species in the study; the greater the resistance, the greater the rate of emissions.

Antioxidant production and its role in protecting four tropical tree species, *Z. mauritiana*; *S. cumini*, *A. indica*, and *M. indica* from air pollution was studied

by Rao and Dubey [74]. Four exposure sites were selected downwind from an industrial source with an average SO_2 concentration of 48–90 $\mu g\,m^{-3}$, while the site 10 km away in upwind direction served as control. Samples were collected once a month for 12 months. SO_2 was the primary pollutant in the area affecting plant response alone and in combination with other pollutants. Sulfate accumulation in the leaves corresponded to the ambient SO_2 level. When exposed to 90 $\mu g\,m^{-3}$ SO_2, the sulfate content of leaves increased by 72, 69, 65, and 92% for *Z. mauritiana*, *S. cumini*, *A. indica*, and *M. indica*, respectively, in comparison to the control site. Increase in sulfate content of four species ranged from 26–48% at the site with an ambient level of SO_2 (48 $\mu g\,m^{-3}$). Stomatal conductance decreased by 26–28% in the four species at the site with the highest SO_2 level in comparison to the control site. Oxidation of proteins, superoxide dismutase activities, and peroxidase activities increased in all four species. The magnitude of the response varied with species and was related to the ambient SO_2 concentration. It was concluded that increased peroxidase and superoxide dismutase activities could increase SO_2 tolerance under field conditions. *Z. mays* was grown for 2 weeks in a fumigation chamber and exposed to 45, 70, and 110 $\mu l\,l^{-1}$ of SO_2. No visible symptoms on plants exposed to charcoal-filtered air were recorded, but differences occurred between the control and the fumigated plants. The amount of cysteine and free amino acids in leaf increased, while that of soluble proteins decreased. Qualitative and quantitative differences found in the soluble protein patterns suggested that low concentrations of SO_2 affect the protein metabolism [124]. Gupta *et al.* [125] studied the effects of SO_2 exposure on ABA production in soybean (*G. max* L.) cv. Elf at the end of the exposure period and after a recovery period of 18 h. Exposure of 30 day-old soybean seedlings to 131, 524, or 1048 $\mu g\,m^{-3}$ of SO_2 for 1, 2, or 4 h resulted in no visible injury at a 131 $\mu g\,m^{-3}$ of SO_2. Although, a mild chlorosis occurred on top leaves after the 18 h recovery period in the 524 $\mu g\,m^{-3}$ SO_2-treated plants. Leaf curl and necrotic areas were visible in plants exposed to 1048 $\mu g\,m^{-3}$ of SO_2 within 4 h of treatment. The authors found both the exposure concentration and duration significantly increased ABA content of leaves. At SO_2 concentration of 131 $\mu g\,m^{-3}$, ABA content increased by 28% after 1 h, 87% after 2 h, and 141% after 4 h exposure. The 18 h recovery period resulted in a reduction in ABA levels in all treatments, but ABA levels were still higher than the controls.

Long-term effects of 39, 73, and 100 $\mu g\,m^{-3}$ of SO_2 (seasonal means) on nitrate reductase, nitrite reductase, glutamine synthetase, glutamate dehydrogenase, and glutathione reductase activity and total glutathione content of winter barley (*H. vulgare* L.) cv. Igri were studied in an open-air fumigation system by Borland and Lea [126]. Nitrate reductase activity in tissues harvested in February, March, and April was significantly decreased by 100 $\mu g\,m^{-3}$ of SO_2. Nitrite reductase activity was relatively constant except for significant increases in April (at 100 $\mu g\,m^{-3}$ of SO_2) and May (at 39 $\mu g\,m^{-3}$ of SO_2). There was no effect of any SO_2 concentration on glutamine synthetase or glutathione reductase. Exposure to SO_2 significantly increased glutamate dehydrogenase activity in samples obtained in December, January, and June. Total glutathione varied with the season, but there was no increase in accumulation on SO_2 exposure. The role of antioxidants and enzymes in metabolic

processes of two pea cultivars, Progress (insensitive) and Nugget (sensitive), which are known to differ in their sensitivity to SO_2, was investigated by Madamanchi and Alscher [127]. Plants were exposed in continuously stirred tank reactors to 2095 μg m^{-3} of SO_2 for 210 min. Total glutathione (ratio of exposed/control) content increased from 1.11 (at 0 min) to 2.04 (at 210 min exposure) in the cv. Progress and from 1.42 (at 0 min) to 1.69 (at 210 min exposure) in the cv. Nugget. Reduced GSH increased in the cv. Progress from 1.11 to 1.93 and in Nugget from 1.37 to 1.59 for 0 and 210 min exposure, respectively. No significant effects were found on ascorbic acid or oxidized glutathione content. Superoxide dismutase activity increased by 90% in Progress, but was unaffected in Nugget. Mean glutathione reductase activity increased by 35 and 21% in cv. Progress and Nugget, respectively. The authors suggested that the significantly increased glutathione content, glutathione reductase, and superoxide dismutase activities in cv. Progress might be a part of its metabolic resistance to SO_2 exposure. Impact of SO_2 on SOD and the ascorbate–glutathione cycle was investigated in a tolerant (cv. Punjab-1) and a sensitive cultivar (cv. JS 7244) of soybean (*G. max* (L.) Merr.) [128]. Despite SO_2 stimulation, SOD activities in cv. JS 7244 increased significantly. This differential response was attributed to the ability of cv. Punjab-1 to enhance glutathione reductase (GR) activity and to maintain high GSH/GSSG and ASA/DHA ratios. Postfumigation analysis indicated that cv. Punjab-1 was able to maintain SO_2-enhanced antioxidants, while they declined in cv. JS 7244 the moment fumigation was terminated. Exposure of SO_2-acclimated plants (cv. Punjab-1) with their enhanced antioxidants to 250 μg m^{-3} of SO_2 for 6 h exhibited no enhanced cellular injury (MDA content) compared to control plants with their normal antioxidant levels. The results indicated the existence of a relationship between the plants' ability to maintain the reduced GSH and ascorbate (ASA) levels and the SO_2 tolerance, to tolerate SO_2-induced oxygen-free radical toxicity with elevated antioxidants.

Changes in thylakoid proteins and antioxidants in two wheat cultivars, namely, Mec and Chiarano with different sensitivity to SO_2, were studied [129]. It was found that thylakoid protein composition depends on a differential ability of the cultivars to maintain elevated levels of ascorbic acid rather than on increasing detoxifying enzyme activities. Bernardi *et al.* [130] studied levels of soluble leaf proteins and the response of the SOD complex of bean plants (*P. vulgaris* L.) cv. Groffy after exposure to SO_2 at 79, 157, or 236 μg m^{-3} for 2, 4, or 7 days. No visible injury symptoms were observed in any of the treated plants. Newly synthesized polypeptides were detected in all treatments and there were quantitative differences between the control and the treated plants for six other protein subunits. The observed changes in protein synthesis were linked to a SO_2 resistance. In addition, SO_2 exposure induced the activation of an additional SOD isoform, which when tested exhibited the characteristics of an iron superoxide dismutase (FeSOD). The authors summarized that the increased activity of the FeSOD was the initiation of activation of the antioxidant system in response to radical formation due to SO_2 oxidation. Jeyakumar *et al.* [80] reported that stomatal frequency and stomatal index of maize seedling cv. Co-1 were not affected when exposed to LD_{50} of SO_2. However, the size of the stomata was significantly reduced and there was also reduction in the amount of

starch and sugar in the stressed plants compared to control. Amylase activity and proline contents were increased in response to SO_2 stress. SO_2 is highly damaging to rice, *O. sativa* japonica-type cv. Nipponbare, and triggers multiple events linked to defense/stress response [41]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immune blotting analysis revealed induction of ascorbate peroxidase(s) (APX) and changes in cysteine proteinase inhibitors (phytocystatus) like proteins. Two-dimensional electrophoresis (2DE) followed by amino acid sequencing also revealed several changes in the 2DE protein profiles of SO_2 fumigated leaves. Most prominent changes in leaves were the induced accumulation of a pathogenesis-related (PR) class 5 (OsPR5) proteins, three PR 10 class proteins (OsPLr10s), ATP-dependent CLP protease, and an unknown protein. Mass spectrometry analysis revealed production of phytoalexins, sakuranetin, and momilactone A in SO_2-stressed leaves. Hao *et al.* [131] studied the responses of superoxide anion radical O_2 and antioxidant enzymes of wheat to SO_2 exposure by introducing gas at different concentrations into the culture boxes. When the concentration of SO_2 was 10 and 40 $\mu l\,l^{-1}$, the O_2 content and peroxidase (POD) and catalase (CAT) activities of wheat leaves were increased, while the activity of SOD was reduced. At 50 $\mu l\,l^{-1}$ of SO_2, the activities of POD and CAT were reduced. On the uppermost leaves, necrosis appeared and more fungi multiplication was recorded on green leaves. Presoaking of wheat seeds with 1 $mmol\,l^{-1}$ salicylic acid (SA) for 6 h or with 10 $mmol\,l^{-1}$ of H_2O_2 for 12 h alleviated the oxidative stress caused by SO_2, as the O_2 content decreased and the activities of antioxidant enzymes increased. Under SO_2 fumigation, ethylene significantly inhibited the activities of test enzymes and promoted the O_2 production rate. With simultaneous application of SA and ethylene, SA almost completely eliminates the influences of ethylene on O_2 production and enzyme activities.

The facultative halophyte *Mesembryanthemum crystallinum* shifts its mode of carbon assimilation from C3 pathway to crassulan acid metabolism (CAM) in response to factors generating reactive oxygen species (ROS) in cells [132]. Exogenous application of SO_2 to *M. crystallinum* plants was employed to assure the role of ROS production in CAM induction. The finding suggests that oxidative stress caused by SO_2 fumigation was not sufficient enough to induce functional CAM. Further evaluation of the influence of SO_2 fumigation/sulfite incubation on the activity and level of SOD isoenzymes, especially FeSOD, which is one of the first indicators correlated with the X_3/CAM transformation, revealed that the activity of FeSOD and SOD CuZn isoforms increased under SO_2/sulfite stress. The pattern of FeSOD and CuZnSOD is probably due to the action of sulfite per second. Tseng *et al.* [133] explored the possibility of overcoming the highly phytotoxic effect of SO_2 and salt stress, by introducing the maize Cu/Zn SOD and/or CAT genes into chloroplasts of Chinese cabbage (*B. campestris* L. ssp. *Pekinensis* cv. Tropical Pride) (referred to as SOD, CAT and SOD + CAT plants). SOD + CAT plants showed enhanced tolerance to 400 ppb SO_2 and visible damage was one-sixth of that in wild-type (CK) plants. Moreover, when SOD + CAT plants were exposed to a high salt treatment of 200 mM NaCl for 4 weeks, the photosynthetic activity of the plants decreased by only 6%, where as that of CK plants by 72%. SOD plants had higher total APX and GR activities than CK plants. SOD plants showed protection against SO_2 and salt stress that were

moderately improved compared to CK plants. However, in CAT plants there was inhibition of APX activity limiting tolerance to stress. Moreover, SOD + CAT plants accumulated more K^+, Ca^{2+}, and Mg^{2+} and less Na^+ in their leaves compared to those of CK plants. The results suggest that the expression of SOD and CAT simultaneously is suitable for the introduction of increased multiple stress protection. In an attempt to improve the tolerance of plants to the toxicity of reactive oxygen species produced in the presence of SO_2, Tseng *et al.* [134] engineered transgenic Chinese cabbage (*B. campestris* L. ssp. *Pekinensis* cv. Tropical Pride) by infection with individual strains of *Agrobacterium* (LBA44o4), each carrying a distinct disarmed T-DNA containing *Escherichia coli* SOD and/or CAT gene(s). Transgenic lines were examined by polymerase chain reaction, Northern blot hybridization, and enzyme activity determination. The study revealed that the frequency of cotransformation with two T-DNAs was greater than 40%. Enhancement of either SOD or CAT activity individually had only a minor effect on 40 $\mu g\ ml^{-1}$ of SO_2 tolerance. Mostly, cotransformed strains that overexpressed both SOD and CAT had high resistance to SO_2. Further analyses showed that not only the activities of SOD and CAT but also the activities of total antioxidant enzymes, such as ascorbate peroxidase and glutathione reductase (GR), were higher in transgenic plants treated with SO_2 than in treated wild-type plants, indicating that the ability to eliminate ROS in transgenic Chinese cabbage was increased significantly. It has been concluded that the cotransformation systems could serve as a good method for plant improvement.

6.3.9
Genotoxicity

Vascular plants are endowed with a useful genetic system for screening and monitoring environmental pollutants. Mutagenic activity of toxic chemicals has been analyzed with different plant systems such as *Allium cepa*, *V. faba*, *Arabidopsis thaliana*, and *H. vulgare*, where chromosomal aberration assays, mutation assays, and cytogenetic tests were performed [135–140]. Plant bioassays, which are considerably sensitive and simple in contrast with animal bioassays, have been authenticated in the international collaborative studies under the United Nations Environment Program (UNEP), World Health Organization (WHO), and US Environmental Protection Agency (US-EPA) and confirmed to be efficient tests for genotoxicity monitoring of environmental pollutants [141, 142]. Sulfur dioxide, as a ubiquitous gaseous air pollutant, influences both human health and the global ecological systems of animals and plants [143]. Numerous studies have shown that SO_2 or its hydrated forms (bisulfite and sulfite) caused visible foliar injury/damage such as chlorosis and necrosis [143], inhibited seedling growth and cell division [144], impaired photosynthetic process [145], and also influenced the activities of enzymes for scavenging reactive oxygen species in plant cells [143]. However, there is rather inadequate information in relation to genotoxic effect of SO_2 in plants [136, 146, 147].

Yi and Meng [147] investigated the genotoxic effect of SO_2 using *A. stavium* and *V. faba* cytogenetic tests (a highly sensitive and simple plant bioassay), by treating a

mixture of sodium bisulfite and sodium sulfite (1 : 3), at various concentrations from 1×10^{-4} to 2×10^{-3} M. The study revealed that genotoxicity expressed in terms of anaphase aberration (AA) frequencies in the *Vicia*-AA test and in terms of micronuclei (MCN) frequencies in both the tests. On average, a 1.7–3.9-fold increase in AA frequencies and a 3.5–4.5-fold increase in MCN frequencies in *Vicia* root tips was observed compared to the negative control. Similarly, *Allium* root tips also showed a significant increase in MCN frequencies in the treated samples and pycnotic cells (PNCs) appeared in the treated groups as well. The frequencies of MCN, AA, and PNC increased independent of doses and the cell cycle delayed at the same time in bisulfite-treated samples. The authors concluded that the *Vicia* and *Allium* cytogenetic bioassays are efficient, simple, and reproducible in genotoxicity studies of bisulfite. Verge *et al.* [148] conducted two experiments (1981 and 1997) and estimated the genotoxic effects of the atmosphere of the industrial estate South of Toulouse, using tobacco plants (heterozygous for two independent *loci* involved in the chlorophyll parenchyma differentiation), on the basis of cellular rate of reversion, which was counted and calculated from the somatic spots of green cellular colonies on yellow green background. The authors observed a general decrease in genotoxic effects and construed it as due to a general decrease in air pollution evaluated by the development of the concentrations of three toxic gases before and after the implementation of cleanup devices. It has been suggested that this bioindicator is efficient, easy to use, and capable of integrating, *in situ*, genotoxic variations throughout the duration of plant growth and development. Longauera *et al.* [149] studied the effects of air pollution on the genetic structure of Norway spruce, European silver fir, and European beech at four polluted sites in Slovakia, Romania, and Czech Republic, and the genotypes of sampled trees were determined at 21 isozyme gene loci of spruce, 18 loci of fir, and 15 loci of beech. The results revealed that in comparison to Norway spruce, fewer genetic differences were in beech and almost no differentiation between pollution-tolerant and -sensitive trees in fir. In adult stands of Norway spruce, sensitive trees exhibited higher genetic multiplicity and diversity. The authors suggested that the decline of pollution-sensitive trees may result thus in a gradual genetic depletion of pollution-exposed populations of Norway spruce through the loss of less frequent alleles with potential adaptive significance to altered stress regimes in the future. Comparison of the subsets of sensitive and tolerant Norway spruce individuals, as determined by the presence or absence of discolorations ("spruce yellowing"), revealed different heterozygosity at 3 out of 11 polymorphic loci.

6.3.10
Sulfur Deficiency

Sulfur is an essential macronutrient for normal plant growth and development. During the last decades, sulfur availability in soils has become the major limiting factor for plant production in many developed countries due to significant reduction in anthropogenic sulfur dioxide emission forced by introduction of stringent environmental legislations. Ironically, it is a result of the positive phenomenon,

namely, a strong reduction in atmospheric pollution in industrialized areas of developed countries [9]. The main cause is the fall in the amount of coal used in electricity generation. Introduction of new technologies such as flue gas desulfurization and "gas reburn" has also contributed to the fall. Beside coal combustion, the biggest source of sulfur dioxide emissions is the combustion of petroleum. However, this has also fallen considerably due to large reductions in fuel oil use in favor of gas in electricity generation and a general option of gas as the fuel of choice for industries. The already small amount of sulfur dioxide emitted from motor spirit and diesel fuel has also fallen and is believed to fall further due to the introduction of ultralow sulfur petrol and diesel. In a majority of the European countries, including Poland, emission has fallen by more than 60% in the years 1990–2004 [150], whereas in Asia these trends are still reverse compared to Europe and the United States. Decreased atmospheric sulfur deposition on agricultural land due to the reduction in sulfur dioxide emission to the atmosphere and the utilization of sulfur-free (however, rich in nitrogen and phosphorus) fertilizers have led to insufficient sulfur supply to a variety of crops, especially those with high sulfur requirements such as oilseed rape [9].

Inadequate sulfate nutrition leads to reduced plant growth, vigor, and resistance to abiotic and biotic stresses [151–153]. Sulfur deficit influences not only the crop yield but also the food quality. For instance, certain sulfur-rich proteins in wheat determine the baking quality of flour [154, 155] and malting quality of barley [156]. A decreased sulfur content in wheat may increase the level of carcinogenic acrylamide in processed food [157]. Furthermore, a sufficient metabolic supply of sulfur amino acids from diet and tissue protein breakdown is necessary for the normal functioning of animal organs, including the mammalian immune system [158, 159]. Sulfur deficiency that decreases the level of sulfur-containing defense compounds, such as elemental sulfur, H_2S, glutathione, phytochelatins, various secondary metabolites, and sulfur-rich proteins, is clearly associated with a decreased resistance of plants, while sulfur fertilization increases their resistance against biotic and abiotic stresses, and this phenomenon is known as sulfur-induced resistance (SIR) [153, 160]. Conversely, sulfur metabolism is also influenced by both the abiotic stresses (which increases the ROS formation) and the oxidative stress. The biochemistry of sulfur assimilation is well characterized; however, many questions remain unsolved concerning regulation of sulfur metabolism in response to both the availability of sulfur in the environment and the increased demand of plants for sulfur metabolites under certain environmental conditions [9].

Numerous studies have demonstrated and it is now a well-documented fact that plants growing in sulfur-deficient soils can benefit by taking up sulfur from the atmosphere during chronic exposures [27, 161–163]. The ability of plants to accumulate atmospheric sulfur is species specific; for example, cotton is more efficient than tall fescue in accumulating atmospheric sulfur [164]. Moreover, nitrogen supply in the soil also has an influence on the degree of the positive growth response to SO_2. This positive growth response will vary accordingly with the nitrogen supply in the soil; that is, being low under low nitrogen and high under sufficient nitrogen [165]. Undoubtedly from a mechanistic point of view, exposure to SO_2 can

be used for sulfur nutrition and to counteract SO_4^{2-} uptake through the roots and transport to the shoots. This way, the negative effects of SO_2 absorption by the shoot and the resulting acidification and excess sulfur accumulation in the foliage may be reduced [166].

6.4 Conclusions

Among all the gaseous air pollutants, SO_2 was the first to be designated as a phytotoxic air pollutant, with that the most important one, and its effects on plants have been the most extensive and longest studied subject in this field. However, since the past few decades it has attracted less attention because of its declined concentrations in the atmosphere in much of the developed countries due to the stringent environmental legislations introduced. Nonetheless, it presents a potential threat in other developing and underdeveloped countries that are still facing its adverse effects on vegetations and agricultural crops resulting in low crop production.

Numerous studies have been undertaken to evaluate crop responses to SO_2. Since SO_2 is an accumulative pollutant in plant tissue, high concentrations of SO_2 can cause acute injury in the form of foliar necrosis, even after relatively short duration exposure. However, in the field such effects are far less important than chronic injury, which results from long-term exposure to much lower concentrations and is essentially cumulative in nature, resulting in reduced growth and yield and increased senescence, often with no clear visible symptoms or else with some degree of chlorosis. Hitherto, much knowledge has been gained on the mechanism of foliar injury and responses of plants to SO_2 exposures. Present efforts to overcome SO_2 stress comprise detoxification processes in plants and evolution of resistance. A few studies have also addressed the beneficial effects of SO_2 on plant growing in sulfur-deficient soils. That being said, other pollutants that are present in the atmosphere can also influence the effect of SO_2 on plants. Thus, the effects of a specified dose of SO_2 can be modified by prevailing environmental conditions. Conversely, SO_2 can also modify the response of plants to other environmental stresses, both biotic and biotic, often intensifying their adverse impacts.

Dose–response relationships have been investigated for various crop plants. The information used in deriving such relationships have been on controlled fumigations, under quasi-field or defined environmental conditions, from filtration experiments and from field studies such as transects along SO_2 gradients. In many of these studies, SO_2 has been considered the sole factor governing plant growth and productivity. For this reason, field and filtration studies provided data on responses under realistic conditions, but these data are confounded by the presence of other pollutants and variable environmental conditions. Nevertheless, reasonably accurate values for no-response thresholds for adverse effects have been derived for broad categories of plants. Though, in the past few decades this perception has changed to a more holistic approach, which includes the joint effects of multiple air pollutants, plant growth-regulating climatic factors, pathogens, and insect pests. To advance our

knowledge and understanding of this subject, research in the future needs to execute this holistic approach and it requires interdisciplinary cooperation among scientists from multiple areas of specialization.

References

1 Khan, M.R. and Khan, M.W. (1993a) *Environ. Pollut.*, **81**, 91–102.
2 Gupta, V., Tomer, Y.S., and Prakash, G. (1993) *Acta Bot. Ind.*, **21**, 230–237.
3 Kumar, N. and Singh, V. (1985) *Ind. J. Ecol.*, **12**, 183–188.
4 Varshney, C.K. and Garg, J.K. (1979) Plant response to sulphur dioxide, in *Critical Reviews in Environmental Control*, CRC Press, Boca Raton, FL, pp. 27–29.
5 Khan, M.W. and Khan, M.R. (1993b) Relationship of plant pathogenic microbes with air pollution, in *Frontiers in Applied Microbiology*, vol. **IV** (eds M.G. Mukerji and V.P. Singh), Rastogi Publishers, Meerut, India, pp. 114–125.
6 Darrall, N.M. (1989) *Plant Cell Environ.*, **12**, 1–30.
7 De Kok, L.J. (1990) Sulphur metabolism in plants exposed to atmospheric sulphur, in *Sulphur Nutrition and Sulphur Assimilation in Higher Plants; Fundamental, Environmental and Agricultural Aspects* (eds H. Rennenberg, C. Brunold, L.J. De Kok, and I. Stulen), SPB Academic Publishing, The Hague, The Netherlands, pp. 111–130.
8 Komarnisky, L.A., Christopherson, RJ., and Basu, TK. (2003) *Nutrition*, **19**, 54–61.
9 Lewandowska, M. and Sirko, A. (2008) *Acta Biochim. Polon.*, **55** (3), 457–471.
10 Agrawal, M. and Verma, M. (1997) *J. Environ. Manage.*, **49**, 231–244.
11 Cape, J.N., Fowler, D., and Davison, A. (2003) *Environ. Int.*, **29**, 201–211.
12 Weil, E.D. and Sandler, S.R. (1997) Sulfur compounds, in *Kirk–Othmer Encyclopedia of Chemical Technology*, John Wiley & Sons, Inc. (on-line edition, accessed 31 August 2002).
13 Hazardous Substances Data Bank (HSDB) (2002) Hazardous Substances Data Bank Sulfur Dioxide (HSN 228), Toxicology and Environmental Health Information Program, National Library of Medicine, Bethesda, MD, available online at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.
14 IARC (1992) Abstract: Sulfur dioxide and some sulfites, bisulfites and metabisulfites, IARC Working Group. TA: IARC Monographs on the Evaluation of Carcinogenic Risk to Human, 54, pp. 131–188.
15 WHO (1979) *Sulfur Oxides and Suspended Particulate Matter, Environmental Health Criteria 8*, World Health Organization, Geneva.
16 Friend, J.P. (1973) The global sulfur cycle, in *Chemistry of the Lower Troposphere* (ed. S.I. Rasool), Plenum Press, New York, NY, pp. 177–201.
17 Agency for Toxic Substances and Disease Registry (ATSDR) (1998) *Toxicological Profile for Sulfur Dioxide*, ATSDR, Public Health Service, US Department of Health and Human Services, Atlanta, GA, December 1998, p. 185.
18 Watsan, R.T., Rodhe, H., Oeschger, H., and Siegenthaler, U. (1990) Greenhouse gases and aerosols, in *Climate Change: The IPCC Scientific Assessment* (eds J.T. Houghton, G.J. Jenkins, and J.J. Ephraums), Cambridge University Press, Cambridge, Great Britain, pp. 1–40.
19 Yunus, M., Singh, N., and Iqbal, M. (1996) Global status of air pollution: an overview, in *Plant Response to Air Pollution* (eds M. Yunus and M. Iqbal), John Wiley & Sons, Ltd, Chichester, UK, pp. 1–34.
20 Innes, J.L. and Haron, A.H. (2000) Air pollution and the forests of developing and rapidly industrializing regions. Report No. 4 of the IUFRO Task Force on Environmental Change. CAB International, Cambridge University Press, Cambridge, UK.

21 Khan, M.R. and Khan, M.W. (2000) Sulphur dioxide effects on plants and pathogens, in *Environmental Hazards, Plant and People*, CBS Publishers & Distributors, Darya Ganj, New Delhi, pp. 118–136.

22 Khan, M.R. and Khan, M.W. (1994a) *New Phytol.*, **126**, 337–345.

23 Khan, M.R. and Khan, M.W. (1994b) *Plant Pathol.*, **43**, 683–693.

24 Barret, R.W. and Benedict, H.M. (1970) Sulphur dioxide, in *Recognition of Air Pollution Injury: A Pictorial Atlas* (eds J.A. Jacobson and A.C. Hill), Air Pollution Control Association, Pittsburgh, Pennsylvania, USA.

25 Legge, A.H. and Krupa, S.V. (2002) Effects of sulphur dioxide, in *Air Pollution and Plant Life* (eds J.N.B. Bell and M. Treshow), John Wiley and Sons, Ltd, West Sussex, England, pp. 135–162.

26 Pell, E.J. (1979) How air pollutants induce disease, in *Plant Disease: An Advanced Treatise*, vol. **IV** (eds J.G. Horsfall and E.B. Cowling), Academic Press, New York, pp. 273–292.

27 Thomas, M.D., Hendrick, R.H., Collier, T.R., and Hill, G.R. (1943) *Plant Physiol.*, **18**, 345–371.

28 Jacobson, S. and Hill, A. (1970) in *Recognition of Air Pollution Injury: A Pictorial Atlas* (eds J.A. Jacobson and A.C. Hill), Air Pollution Control Association, Pittsburgh, Pennsylvania, USA.

29 Legge, A.H., Jager, H.-J., and Krupa, S.V. (1998) Sulphur dioxide, in *Recognition of Air Pollution Injury to Vegetation: A Pictoral Atlas*, 2nd edn (ed. R.B. Flagler), Air and Water Management Association, Pittsburgh, PA, pp. 3-1–3-42.

30 Smith, W.H. (1990) *Air Pollution and Forests*, 2nd edn, Springer, New York, NY.

31 Krupa, S.V. (1996) The role of atmospheric chemistry in the assessment of crop growth and productivity, in *Plant Response to Air Pollution* (eds M. Yunus and M. Iqbal), John Wiley & Sons, Ltd, Chichester, UK, pp. 35–73.

32 Bell, J.N.B. (1982) Sulphur dioxide and growth of grasses, in *Effects of Gaseous Pollutants in Agriculture and Horticulture* (eds M.H. Unsworth and D.P. Ormrod), Butterworth Scientific, London, UK, pp. 225–246.

33 Legge, A.H., Nosal, M., and Krupa, S.V. (1996) *Can. J. Forest Res.*, **26**, 689–695.

34 USNRC (National Research Council) (1989) *Biologic Markers of Air Pollution Stress and Damage in Forests*, National Academy Press, Washington DC.

35 Karnosky, D. (1985) Genetic variability in growth responses to SO_2, in *Sulphur Dioxide and Vegetation: Physiology, Ecology and Policy Issues* (eds W.E. Winner, H.A. Mooney, and R.A. Goldstein), Stanford University Press, Stanford, CA, pp. 346–356.

36 Tingey, D.T. and Olszyk, D.M. (1985) Intraspecies variability in metabolic responses to SO_2, in *Sulphur Dioxide and Vegetation: Physiology, Ecology and Policy Issues* (eds W.E. Winner, H.A. Mooney, and R.A. Goldstein), Stanford University Press, Stanford, CA, pp. 179–205.

37 Shaw, P.J.A., Holland, M.R., Darrall, N.M., and McLeod, A.R. (1993) *New Phytol.*, **123**, 143–152.

38 Clapperton, M.J. and Reid, D.M. (1994) *Environ. Pollut.*, **86**, 251–258.

39 Padhi, S.K., Rath, S., and Kar, M. (1995) *Orissa J. Horticul.*, **23**, 119–122.

40 Rath, S., Padhi, S.K., Kar, M., and Ghosh, P.K. (1995) *J. Orna. Horticul.*, **2**, 42–45.

41 Rakwal, R., Agrawal, G.K., Kubo, A., Tamogami, S., and Iwahashi, H. (2003) *Environ. Exp. Bot.*, **49**, 223–235.

42 Khan, M.R., Khan, M.W., and Singh, K. (1995) *Nematol. Medit.*, **23**, 9–13.

43 Khan, M.R. and Khan, M.W. (1996a) *Nematol. Medit.*, **24**, 33–35.

44 De Kok, L.J., Castro, A., Durenkamp, M., Stuiver, C.E.E., Westerman, S., Yang, L., and Stulen, I. (2002) Sulphur in plant physiology. Proceedings No. 500, International Fertiliser Society, York, UK pp. 1–26.

45 De Kok, L.J., Castro, A., Durenkamp, M., Koralewska, A., Posthumus, F.S., Stuiver, C.E.E., Yang, L., and Stulen, I. (2005) *Landbouforschung Volkenrode*, **283**, 176–185.

46 Hawkesford, M.J. and Wray, J.L. (2000) *Adv. Bot. Res.*, **33**, 159–223.

47 Buchner, P., Stuiver, C.E.E., Westerman, S., Wirtz, M., Hell, R., Hawkesford, M.J., and De Kok, L.J. (2004) *Plant Physiol.*, **136**, 3396–3408.

48 Wilson, S.A. and Murray, F. (1990) *Environ. Pollut.*, **66**, 179–191.

49 Van Der Kooij, T.A.W., De Kok, L.J., Haneklaus, S., and Schnug, E. (1997) *New Phytol.*, **135**, 101–107.

50 Stulen, I., Perez-Soba, M., De Kok, L.J., and Van der Eerden, L.J. (1998) *New Phytol.*, **139**, 61–70.

51 Cram, W.J. (1990) Uptake and transport of sulphate, in *Sulphur Nutrition and Sulphur Assimilation in Higher Plants* (eds H. Rennenberg, C. Brunold, L.J. De Kok, and I. Stulen), SPB Academic Publishing, The Hague, The Netherlands, pp. 3–11.

52 Clarkson, D.T., Hawkesford, M.J., and Davidian, J.C. (1993) Membrane and long distance transport of sulphate, in *Sulphur Nutrition and Assimilation in Higher Plants: Regulatory Agricultural and Environmental Aspects* (eds L.J. De Kok, I. Stulen, H. Rennenberg, C. Brunold, and W.E. Rauser), SPB Academic Publishing, The Hague, The Netherlands, pp. 3–19.

53 De Kok, L.J. and Tausz, M. (2001) The role of glutathione in plant reaction and adaptation to air pollutants, in *Significance of Glutathione to Plant Adaptation to the Environment* (eds D. Grill, M. Tausz, and L.J. De Kok), Kluwer Academic Publishers, Dordrecht, pp. 185–201.

54 De Kok, L.J., Stuiver, C.E.E., and Stulen, I. (1998) Impact of atmospheric H_2S on plants, in *Responses of Plants Metabolism to Air Pollution and Global Change* (eds L.J. De Kok and I. Stulen), Backhuys Publishers, Leiden, The Netherlands, pp. 51–63.

55 Lendzian, K.L. (1984) Permeability of plant cuticles to gaseous air pollutants, in *Gaseous Air Pollutants and Plant Metabolism* (eds M.J. Koziol and F.R. Whatley), Butterworths, London, U.K, pp. 77–81.

56 Tausz, M., Van der Kooij, T.A.W., Müller, M., De Kok, L.J., and Grill, D. (1998) Uptake and metabolism of oxidized and reduced sulphur pollutants by spruce trees, in *Responses of Plant Metabolism to Air Pollution and Global Change* (eds L.J. De Kok and I. Stulen), Backhuys Publishers, Leiden, The Netherlands, pp. 457–460.

57 Van Der Kooij, T.A.W. and De Kok, L.J. (1998) Kinetics of deposition of SO_2 and H_2S to shoots of *Arabidopsis thaliana* L, in *Responses of Plants Metabolism to Air Pollution and Global Change* (eds L.J. De Kok and I. Stulen), Backhuys Publishers, Leiden, pp. 479–481.

58 Clarke, K. and Murray, F. (1990) *New Phytol.*, **115**, 633–637.

59 Agrawal, M. and Singh, J. (2000) *Environ. Monit. Assess.*, **60**, 261–282.

60 Abdul Wahab, S.A. and Yaghi, B. (2004) *J. Environ. Sci. Health*, **39**, 1559–1571.

61 Yang, L., Stulen, I., and De Kok, L.J. (2006) *Environ. Exp. Bot.*, **57**, 236–245.

62 Dwivedi, A.K., Tripathi, B.D., and Shashi (2008) *J. Environ. Biol.*, **29**, 377–379.

63 Brychkova, G., Xia, Z., Yang, G., Yesbergenova, Z., Zhang, Z., Davydov, O., Fluhr, R., and Sagi, M. (2007) *Plant J.*, **50**, 696–709.

64 Gerini, O., Guidi, L., Lorenzini, G., and Soldatini, G.F. (1990) *J. Environ. Qual.*, **19**, 154–156.

65 Darrall, N.M. (1991) *Agric. Ecosys. Environ.*, **33**, 309–324.

66 Panigrahi, N.C., Mishra, B.B., and Mahoanty, B.K. (1992) *J. Environ. Biol.*, **13**, 201–205.

67 Lorenzini, G., Panicucci, A., and Nali, C. (1995) *Water Air Soil Pollut.*, **85**, 1257–1262.

68 Ranieri, A., Pieruccetti, F., Panicucci, A., Castagna, A., Lorenzini, G., and Soldatini, G.F. (1999) *Plant Physiol. Biochem.*, **37**, 919–929.

69 Swanepoel, J.W., Kruger, G.H.J., and Van Heerden, P.D.R. (2007) *J. Arid Environ.*, **70**, 208–221.

70 Mansfield, T.A. (1998) *Environ. Pollut.*, **101**, 1–11.

71 Khan, M.R. and Khan, M.W. (1996b) *Nematropica*, **26**, 47–56.

72 Han, S. (2001) *Ying Yong Sheng Tai Xue Bao*, **12** (3), 425–428.

73 Dhir, B., Mahmooduzzafar, Siddiqi, T.O., and Iqbal F M. (2001) *J. Plant Biol*, **44**, 97–102.

74 Rao, M.V. and Dubey, P.S. (1990) *Water Air Soil Pollut.*, **51**, 297–305.

75 Wali, B., Iqbal, M., and Mahmooduzzafar (2007) *Flora*, **202**, 268–280.

76 Nieboer, E., Richardson, D.H.S., Puckett, K.J., and Tomassini, F.D. (1976) in *Effects of Air Pollutants on Plants* (ed. T.A. Mansfield), Cambridge University Press, Cambridge, UK, pp. 61–85.

77 Prasad, B.J. and Rao, D.N. (1982) *Environ. Pollut.*, **29**, 57–70.

78 Prakash, G., Gupta, V., Poonia, S., and Sharma, S.S. (2002) *Plant Archives*, **2** (2), 165–170.

79 Wang, Y., Wisniewski, M., Meilan, R., Cui, M., Webb, R., and Fuchigami, L. (2005) *J. Am. Soc. Horticul. Sci.*, **130**, 167–173.

80 Jeyakumar, M., Jayabalan, N., and Arockiasamy, D.I. (2003) *Physiol. Mol. Biol. Plants*, **9**, 147–151.

81 Cameron, C.A. (1874) *Gard. Chron. (London)*, **1**, 274–275.

82 Darley, E.F. (1968) *Phytopatholgy*, **54**, 1075.

83 Haywood, J.K. (1905) *US Dept. Agric. Bur. Chem. Bull.*, **89**, 1–23.

84 Haywood, J.K. (1910) *US Dept. Agric. Bur. Chem. Bull*, **89**, 1–63.

85 Scheffer, T.C. and Hedgcock, G.G. (1955) *US Forest Service Technol. Bull.*, **1117**, 1–49.

86 Carlson, C.E. (1974) Sulphur damage to Douglas fir near a pulp and paper industry in Western Montana, US Forest Serv. Div. State, Priv. Forest Missoula pp. 1–41.

87 Errington, J.C. and Thirgood, J.V. (1971) *Annu. Rev.*, 72–75.

88 Linzon, S.N. (1966) Effect of air born sulphur pollutants on plants, in *Sulphur in the Environment, Part II* (ed. J.O. Nriagu), John Wiley & Sons, Inc., New York, pp. 109–162.

89 Gordon, A.G. and Dorham, E. (1963) *Can. J. Bot.*, **41**, 1063–1078.

90 US EPA (*US Environmental Protection Agency*) (1971) Official pollution abatement activity: Pre-conference report. Mt. Storm, West Virginia Gorman, Maryland Pollution Control Office Publication 1, No. APTD-0656, pp. 21–48.

91 Wood, F.A. (1967) Air pollution and shade trees. Proceedings of the 43rd International Conference Shade Tree, pp. 66–82.

92 Ghouse, A.K.M. and Amani, A.Z. (1978) Effect of air pollution on fruit formation and seed setting in *Dalbergia sissoo*. Proceedings of the International Symposium on "Environmental Agents and their Biological Effects," Hyderabad, India.

93 Ghouse, A.K.M. and Khan, A.U. (1978) Environmental pollution and epidermal structure in *Psidium guajava* L. Proceedings of the International symposium on "Environmental Agents and their Biological Effects," Hyderabad, India.

94 Zaidi, S.H., Amani, A.Z., Farooqui, M.H., and Ghouse, A.K.M. (1979) Leaf epidermal structure of *Croton bonplandianus* Baill in relation to air pollution. Proceedings on Symposium of Environmental Biology, pp. 239–242.

95 Mishra, L.C. (1982) *Environ. Pollut.*, **28**, 281–284.

96 Gupta, M.C. and Ghouse, A.K.M. (1987) *Environ. Pollut.*, **43**, 263–270.

97 Ashenden, T.W. (1987) *Environ. Pollut.*, **45**, 29–47.

98 Khan, M.R., Singh, S.K., and Khan, M.W. (1988) Primary productivity of *Cynodon dactylon* in seminatural grazing lands in relation to air pollution. Proceedings of the 3rd International Rangeland Congress, New Delhi, 1, pp. 93–95.

99 Lorenzini, G., Panicucci, A., and Guidi, L. (1990) *Bull. Environ. Contam. Toxicol.*, **45**, 408–414.

100 Atkinson, C.J., Wookey, P.A., and Mansfield, T.A. (1991) *New Phytol.*, **117**, 535–541.

101 Khan, M.R. and Khan, M.W. (1991) *J. Ind. Bot. Soc.*, **70**, 239–244.

102 Khan, M.W., Khan, M.R., and Khan, A.A. (1991b) *J. Ind. Bot. Soc.*, **70**, 373–378.

103 García, D., Rodríguez, J., Sanz, J.M., and Merino, J. (1998) *Ecotoxicol. Environ. Safety*, **40** (1–2), 42–48.

104 Coleman, J.S., Mooney, H.A., and Winner, W.E. (1990) *Can. J. Bot.*, **68**, 102–106.

105 Weigel, H.J., Adaros, G., and Jager, H.J. (1990) *Environ. Pollut.*, **67**, 15–28.

106 Murray, F. and Wilson, S. (1990) *New Phytol.*, **114**, 537–541.

107 Kropff, M.J. (1990) *New Phytol.*, **115**, 357–365.

108 Murray, F. and Wilson, S. (1991) *Environ. Exp. Bot.*, **31**, 319–325.

109 Qifu, M. and Murray, F. (1991) *New Phytol.*, **118**, 101–109.

110 Colls, J.J., Geissler, P.A., and Baker, C.K. (1992) *Agric. Ecosys. Environ.*, **38**, 3–10.

111 Julkunen-Tiito, R., Lavola, A., and Kainulainen, P. (1995) *Water Air Soil Pollut.*, **83**, 195–203.

112 Singh, K., Khan, M.W., and Khan, M.R. (1995) *J. Ind. Bot. Soc.*, **75**, 49–54.

113 Ashenden, T.W., Hunt, R., Bell, S.A., Williams, T.G., Mann, A., Booth, R.E., and Poorter, L. (1996) *Funct. Ecol.*, **10**, 483–490.

114 Tiwari, S., Agrawal, M., and Marshall, F.M. (2010) *Trop. Ecol.*, **51**, 353–363.

115 Houston, D.B. and Dochinger, L.S. (1977) *Environ. Pollut.*, **12**, 1–5.

116 Pelz, E. (1963) *Archiv f. Forstwesen*, **12**, 1066–1077.

117 Bosac, C., Black, J., Black, C.R., Roberts, J.A., and Lockwood, F. (1993) *New Phytol.*, **124**, 439–446.

118 Agrawal, S., Singh, R., and Sahi, A. (1995) *Cytobios*, **83**, 41–47.

119 Tanaka, K. and Sugahara, K. (1980) *Plant Cell Physiol.*, **21**, 601–612.

120 Pierre, M. and Queiroz, O. (1981) *Environ. Pollut.*, **25**, 41–52.

121 Varshney, S.R.K. and Varshney, C.K. (1985) *Environ. Exp. Bot.*, **25**, 107–114.

122 Chiment, J.J., Alscher, R., and Hughes, P.R. (1986) *Environ. Exp. Bot.*, **26**, 147–152.

123 Chauhan, A. (1990) *Oecologia*, **84**, 289–294.

124 Raneiri, R.A., Bernardi, R., Pisanelli, A., Lorenzini, G., and Soldatini, G.F. (1990) *Plant Physiol. Biochem.*, **28**, 601–607.

125 Gupta, G., Sandhu, R., and Mulchi, C. (1991) *J. Environ. Qual.*, **20**, 151–152.

126 Borland, A.M. and Lea, P.J. (1991) *Agric. Ecosys. Environ.*, **33**, 281–292.

127 Madamanchi, N.R. and Alscher, R.G. (1991) *Plant Physiol.*, **97**, 88–93.

128 Rao, M.V. and Dubey, P.S. (1993) *Environ. Pollut.*, **82**, 99–105.

129 Ranieri, A., Castagna, A., Lorenzini, G., and Soldatini, G.F. (1997) *Environ. Exp. Bot.*, **37**, 125–135.

130 Bernardi, R., Nali, C., Gargiulo, R., Pugliesi, C., Lorenzini, G., and Durante, M. (2001) *J. Phytopathol.*, **149**, 477–480.

131 Hao, L., Zhang, H., Xu, X., Tao, S., and Yu, L. (2005) *Ying Yong Sheng Tai Sue Bao*, **16**, 1038–1042.

132 Surowka, E., Karolewski, P., Niewiadomska, E., Libik, M., and Miszalski, Z. (2007) *Plant Sci.*, **172**, 76–84.

133 Tseng, M.J., Liu, C.W., and Yiu, J.C. (2007) *Plant Physiol. Biochem.*, **45**, 822–833.

134 Tseng, M.J., Liu, C.W., and Yiu, J.C. (2008) *Sci. Hortic. (Amsterdam)*, **115**, 101–110.

135 Conte, C., Mutti, I., Puglisi, P., Ferrarini, A., Regina, G., Maestri, E., and Marmiroli, N. (1998) *Chemosphere*, **37**, 2739–2749.

136 Yi, H.L. and Meng, Z.Q. (2002) *Acta Phytoecol. Sin.*, **26**, 303–307.

137 Ma, T.H., Xu, Z., Xu, C., McConnell, H., Rabago, E.V., Arreola, G.A., and Zhang, H. (1995) *Mutat. Res.*, **334**, 185–195.

138 de Serres, F.J. (ed.) (1994) *Mutat. Res.*, **310**, 167–266.

139 Zaka, R., Chenal, C., and Misset, M.T. (2002) *Mutat. Res.*, **517**, 87–99.

140 Menke, M., Chen, I.P., Angelis, K.J., and Schubert, I. (2001) *Mutat. Res.*, **493**, 87–93.

141 Grant, W.F. (1999) *Mutat. Res.*, **426**, 107–112.

142 Ma, T.H. (1999) *Mutat. Res.*, **426**, 103–106.

143 Noji, M., Saito, M., Nakamura, M., Aono, M., Saji, H., and Saito, K. (2001) *Plant Physiol.*, **126**, 973–980.

144 Yi, H.L. and Meng, Z.Q. (2001) *Bull. Bot. Res.*, **21**, 384–387.

145 Wang, H.W. and Shen, Y.G. (2002) *J. Plant Physiol. Mol. Bio.*, **28**, 247–252.

146 Ma, T.H., Isbandi, D., Khan, S.H., and Tseng, Y.S. (1973) *Mutat. Res.*, **21**, 93–100.

147 Yi, H.L. and Meng, Z.Q. (2003) *Mutat. Res.*, **537**, 109–114.

148 Verge, X., Chapuis, A., Delpoux, M., Dulieu, H., Fabre, A., and Meybeck, M.P. (2004) *Environ. Monit. Assess.*, **91**, 199–209.

149 Longauera, R., Gomoryb, D., Pauleb, L., Bladac, I., Popescud, F., Mankovskaa, B., Muller-Starcke, G., Schuberte, R., Percyf, K., Szarog, R.C., and Karnoskyh, D.F. (2004) *Environ. Pollut.*, **130**, 85–92.

150 Vestreng, V., Myhre, G., Fagerli, H., Reis, S., and Tarrason, L. (2007) *Atmosph. Chem. Phys.*, **7**, 3663–3681.

151 Scherer, H.W. (2001) *Eur. J. Agron.*, **14**, 81–111.

152 Knop, M., Pacyna, S., Voloshchuk, N., Kant, S., Mullenborn, C., Steiner, U., Kirchmair, M., Scherer, H.W., and Schulz, M. (2007) *J. Chem. Ecol.*, **33**, 225–237.

153 Kruse, C., Jost, R., Lipschis, M., Kopp, B., Hartmann, M., and Hell, R. (2007) *Plant Biol.*, **9**, 608–619.

154 Zhao, F.J., Hawkesford, M.J., and McGrath, S.P. (1999) *J. Cereal Sci.*, **30**, 1–17.

155 Granvogl, M., Wieser, H., Koehler, P., Tucher, S.V., and Schieberle, P. (2007) *J. Agric. Food Chem.*, **55**, 4271–4277.

156 Zhao, F.J., Fortune, S., Barbosa, V.L., McGrath, S.P., Stobart, R., Bilsborrow, P.E., Booth, E.J., Brown, A., and Robson, P. (2006) *J. Cereal Sci.*, **43**, 369–377.

157 Muttucumaru, N., Halford, N.G., Elmore, J.S., Dodson, A.T., Parry, M., Shewry, P.R., and Mottram, D.S. (2006) *J. Agric. Food Chem.*, **54**, 8951–8955.

158 Grimble, R.F. (1994) *Adv. Exp. Med. Biol.*, **359**, 41–49.

159 Hunter, E.A. and Grimble, R.F. (1997) *Clin. Sci. (London)*, **92**, 297–305.

160 Rausch, T. and Wachter, A. (2005) *Trends Plant Sci.*, **10**, 503–509.

161 Olsen, R.A. (1957) *Soil Sci.*, **84**, 107–111.

162 Cowling, D.W., Jones, L.H.P., and Lockyer, D.R. (1973) *Nature*, **243**, 479–480.

163 Noggle, J.C. (1980) Sulfur accumulation by plants: the role of gaseous sulfur in crop nutrition, in *Atmospheric Sulfur Deposition: Environmental Impact and Health Effects* (eds D.S. Shriner, C.R. Richmond, and S.E. Lindberg), Ann Arbor Science, Ann Arbor, MI, pp. 289–298.

164 Noggle, J. and John H. (1979) Accumulation of atmospheric sulfur by plants and sulfur supplying capacity of soils. EPA-600/7-79-109. Report prepared by Tennessee Valley Authority, Chattanooga, TN for US Environmental Protection Agency, Washington, DC.

165 Cowling, D.W. and Lockyer, D.R. (1978) *J. Exp. Bot.*, **29**, 257–265.

166 Rennenberg, H. and Herschbach, C. (1996) Responses of plants to atmospheric sulphur, in *Plant Response to Air Pollution* (eds M. Yunus and M. Iqbal), John Wiley & Sons, Ltd., Chichester, UK, pp. 285–294.

7
Excess Soil Phosphorus: Accelerated P Transfer, Water Quality Deterioration, and Sustainable Remediation Strategies

Nilesh C. Sharma and Shivendra V. Sahi

Phosphorus is a limiting nutrient for plant growth. Yet its high accumulation in agricultural soils causes grave environmental concerns affecting human health. The problem is more acute in temperate climate where large farms with intensive animal-based agriculture generate and dump huge amounts of organic litter or manure – a potential source of phosphorus (P). Need for removal of excess P is increasingly felt in recent times giving rise to various remediation strategies: chemical, physical, and biological. Application of chemical amendments, such as lime, ferric chloride, or alum to animal manures or soils enriched with P is one of the methods used commonly in recent times. P immobilization in soil by these amendments may not be stable on a long-term basis. Another strategy to address the excess manure P involves the treatment of animal feed with additives such as phytase and vitamin D that can increase the digestibility of P in diet. Although phytase can decrease total P in litter, it can increase the water-soluble phosphorus in the litter and hence the potential for P losses to surface waters following land application. Likewise, application of biosolids as P fertilizer is also not considered the best management practice from P loss standpoint. Alternatively, phytoremediation, plant-assisted removal of water-soluble P, could be an attractive strategy. Mining of soil P, which includes harvesting P taken up from the soil by a crop grown without external P application, is being examined as one of the crop management strategies for P-impacted soils. It has also been suggested that for the success of P mining, the remedial strategy should include plants that can accumulate P manifold higher (>1% DW) than the P content of common plants. Employing plant species with capabilities like overexpression of root phosphatase and assimilation of organic P from soil P pool could enhance the efficacy of P phytoremediation. Studies suggest that integrating best management practices for all the operations involved in P use, from animal feeding strategies to manure management, to soil and crop management, and to soil P test methods or P loss tools can effectively reduce the environmental risks of diffuse P pollution.

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7.1
Phosphorus Conundrum

Phosphorus (P) content of soil presents a paradoxical scenario in different regions of the world. While many tropical regions with low-input systems of agriculture are faced with low availability of soluble P, some temperate regions with intensive animal-based agriculture have to deal with excessive P in the soil that is threatening the ecosystem.

7.1.1
P Deficiency

The principal form of P in nature is phosphate in inorganic form or bound in ester bonds into organic compounds. Inorganic P (Pi) forms in tropical, relatively unweathered, environments are associated with Ca or Mg. A typical solubility for natural calcium phosphates at neutral pH is near $3\,\mu g\,l^{-1}$. In more weathered soils, which have lost most of their cations, P is increasingly associated with iron (Fe) and aluminum (Al) oxides that further lower P solubility. Soil solution P concentrations vary from 9–36 $\mu g\,l^{-1}$ over a range of tropical soils [1]. This places them well below the P requirement of productive crops ($200\,\mu g\,l^{-1}$), thus creating augmentation needs for P fertilizers. Oxisols cover large areas in the tropics and subtropics, accounting for 50% of the world's P requirements for crop production. The low-input soils of these geographical regions are characterized by high P adsorption capacity and thus a large proportion of total P is converted to P forms that are not available to plants [2]. More than 1 billion ha of soil in the tropics and subtropics exhibit significant P fixation resulting from an increase in the amount of sesquioxides that occurs during the weathering process in Oxisols and Ultisols. It is estimated that 95% of the acidic soils located in tropical Africa, America, Asia, Australia, and the Pacific islands are deficient in P [3].

7.1.2
P Abundance

In many parts of the United States and Europe, where enormous quantities of nutrient-rich manures (chicken, swine litter, and other animal wastes) are spread over the soils, P in manures often exceeds crop requirements [4, 5]. Over last decades, there has been a dramatic shift in the structure of animal production across the United States. Large livestock operations have replaced small and medium-sized operations and have become more concentrated spatially across the United States. This trend has resulted in a huge supply of animal manure for disposal on a limited amount of land area (Table 7.1). Intensive, long-term application rates of manure to soils in these regions have contributed to frequent reports that the quantity of manure nutrients relative to the assimilative capacity of the land has grown out of balance [6, 7]. For example, in Chesapeake Bay region – an area with concentrated poultry production – over 70% of the soils that have been used for long-term poultry waste

Table 7.1 US annual production of manure, nitrogen, and phosphorus from livestock and poultry.

Species	Dry manure million ton	N % of dry manure	P concentration	N thousand ton/annum	P
Swine	15.5	4.7	2.97	730	460
Poultry	15.4	5.13	1.62	790	250
Beef cattle	96.6	3.96	1.07	3828	1029
Dairy cattle	29.1	3.75	0.79	1091	230
Sheep	1.8	3.89	0.56	70	10

Source: Adapted from Ref. [11].

application are now considered to have optimal (no P input needed to maximize yield) or excessive P contents [8]. Sims *et al*. [9] reported that in Sussex Co., DE (USA), which alone produces 230 million broilers and 52 000 head of swine per year, approximately 92% of soils in the county were rated as optimum or excessive in P. The link between high-density animal production and high soil test P (STP) has been clearly established by researchers [6, 9]. Figure 7.1 shows the farm-level excess manure P for the United States [10]. The darker areas represent areas where local crop P requirements are exceeded by local manure P production, and there is concern that these manure P excesses can cause rising STP following land application over a period of years.

Fertilizer P use increased dramatically in the decades following World War II in most developed countries, and over the past few decades, animal production has also become more geographically concentrated. Withers *et al*. [12] reported that the

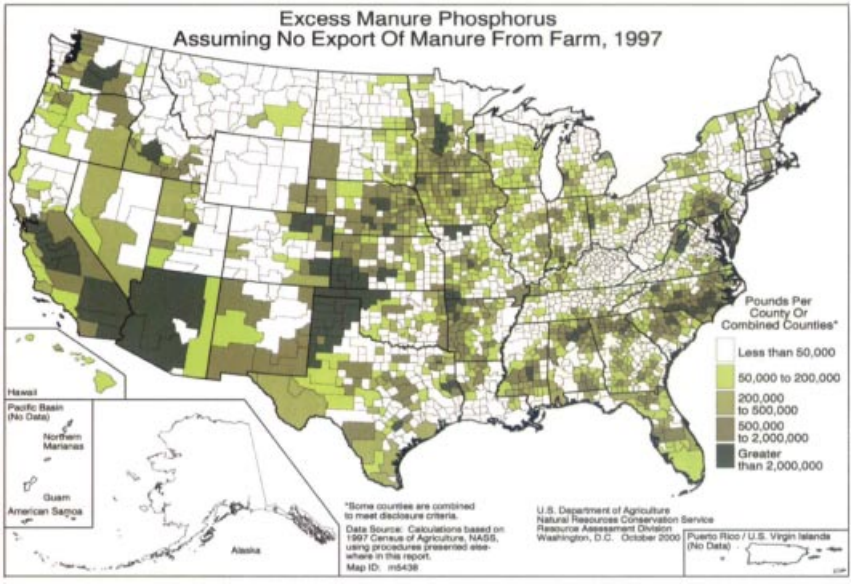

Figure 7.1 Excess manure phosphorus in different counties of US states. Reproduced from Ref. [10], with kind permission from ASA, CSSA, SSSA.

productive grassland and arable areas in the United Kingdom had on average accumulated about 1000 kg P ha^{-1} over the past 65 years. The cumulative mean surplus of German agricultural soils was estimated at 1100 kg ha^{-1} for the period 1952–1990, and for the Netherlands this estimation varied between 1000 and 5000 kg ha^{-1} [13].

7.1.3 Pathways of P Loss

Bulk of runoff P (85–95%) from pastures fertilized with poultry or swine litter is in the soluble form, which is the most readily available form for algal uptake [14]. Surface runoff P is the generous output not only from intensive animal-based agriculture but also from low-input systems of agriculture that augments agricultural fields with P-based fertilizers to boost productivity. Other than overland flow, a potential pathway for P movement is P leaching from soils oversaturated with P. Some 10% of P export from land occurs by leaching and groundwater transport, while 90% is transported by overland flow as sediment or dissolved P. Phosphorus losses by overland transport range from 0.1 to10 kg ha^{-1} yr^{-1} or more on highly erosive sites. On average, surface runoff waters carry 10 μg l^{-1} of dissolved and 1000 μg l^{-1} of sediment P [15, 16]. Several researchers reported that it is not the quantity of P in a soil that determines how much P will be released, but the degree of soil saturation with P that determines the intensity of the solution P concentration a soil can maintain and hence the concentration of P that can be lost in runoff and leaching [17]. Calculating the degree of phosphorus saturation (DPS) of a soil provides a measure to determine whether a soil might leach P. A positive linear relationship occurs between measured soluble P and the DPS of a soil. Studies suggest that STP on its own is a useful indicator of the potential for P losses, but other P loss tools such as the "P index," which incorporate factors such as (i) transport processes that connect a site with surface waters, (ii) management factors, such as manure application and tillage practices, and (iii) proximity to a water body sensitive to P inputs, will be more accurate means of P loss risk assessment [18].

7.1.4 Soil P Dynamics

The development of sustainable land management practices for agroecosystems requires a fundamental understanding of the chemical, biological, and physical processes in soils that affect the availability of P to terrestrial plants, and ultimately to animals and humans [19]. Phosphorus cycling in soils is a complex phenomenon that is strongly influenced by the nature of inorganic and organic solid phases present, type and an extent of biological activity occurring, chemistry of the soil solution (e.g., pH, ionic strength, and redox potential), and many environmental factors such as soil moisture content and temperature (Figure 7.2). Soils, plants, and microorganisms all play a major role. The quantity of P in the soil solution at a given time is generally on the order of <1 kg ha^{-1} or $<1\%$ of the total quantity of P in the soil. This requires that

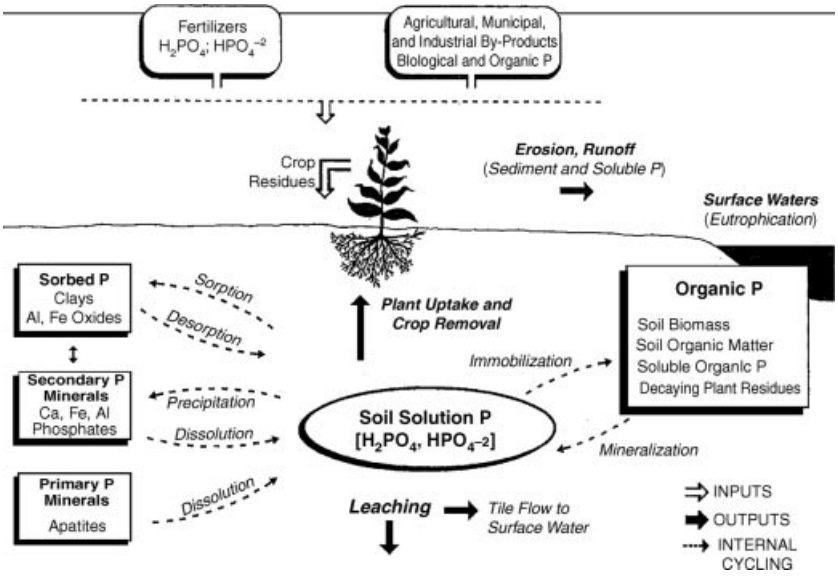

Figure 7.2 The soil P cycle. Reproduced from Ref. [19], with kind permission from with kind permission from ASA, CSSA, SSSA.

the P in the soil solution must be replenished many times over the life of the plant to meet P demands. As the soil interacts with runoff or becomes part of the sediment load in a receiving body of water, the same exchange phenomenon becomes important from an environmental standpoint. "Labile P" is often used to describe soil or sediment P that rapidly equilibrates with an aqueous solution while forms of P that are slow to equilibrate are termed "nonlabile." The mechanisms that control the rate and extent of P exchange between the aqueous and the solid phases in soil or sediment systems are not well known [19].

Maintaining the concentration of P in soil solution in an optimum range for plant growth, often cited as $>200\,\mu g\,l^{-1}$, while restricting P in surface waters to $>30\,\mu g\,l^{-1}$ is a challenge faced both in the agriculture and in the environmental management. This requires to manage the soil P cycle, and the chemical and biochemical processes such as dissolution–precipitation, sorption–desorption, mineralization–immobilization, and oxidation–reduction (Figure 7.2).

Total P concentrations in soil vary in the range of 100–3000 $mg\,kg^{-1}$. Typically, organic P constitutes 30–65% of total P. However, high organic matter soils can contain up to 90% organic P [20]. Organic P transformations in soil are important in determining the overall biological availability of P, which in turn influences ecosystem productivity. The amounts, forms (Table 7.2), and dynamics of organic P in the soil are determined by a combination of biological, chemical, and physical factors (Figure 7.3). The transfer of P in drainage from agricultural soils to watercourses is a key component of the global P cycle and thus has important implications for water

Table 7.2 Percentage distribution of organic phosphorus compounds in soil.

Organic P form	Concentration range (% of total organic P)
Orthophosphate monoesters	
Inositol phosphate	1–100
Sugar phosphates	Trace
Mononucleotides	Trace
Phosphoproteins	Trace
Orthophosphate diesters	
Teichoic acids	0–20
Phospholipids	0–5
Nucleic acids	0–2
Phosphonates	0–12
Organic phosphate anhydrides	Trace

Source: Adapted from Ref. [26].

quality. The enrichment of receiving waters with P can contribute to eutrophication. Organic P is a key component of the P transfer process because it can account for a large proportion of the total P in soil solution, runoff water, and stream water [21]. Some organic P forms are mobile in the soil, leading to penetration of soluble organic P to depth, notably where animal manure is applied to the soil [22]. Organic P is also

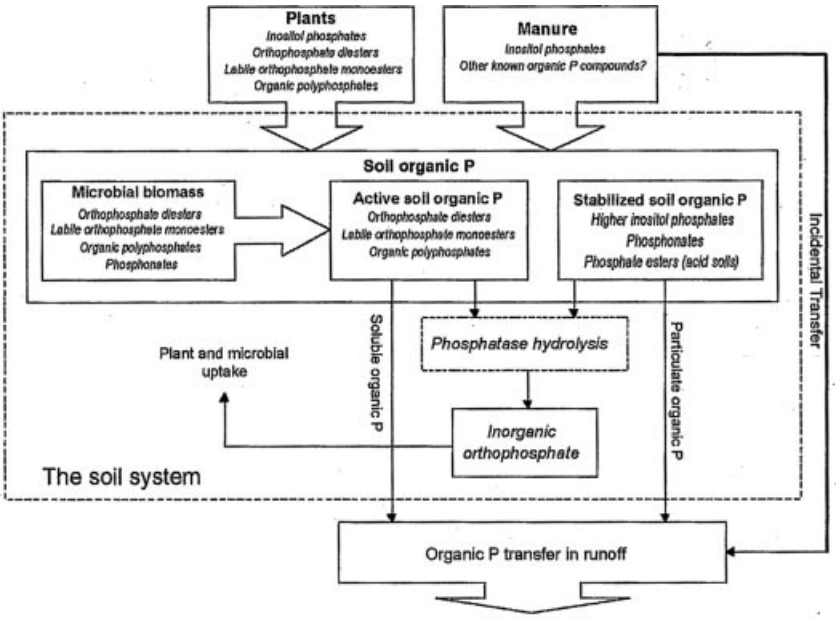

Figure 7.3 Soil organic P dynamics – conceptual model. Reproduced from Ref. [25] with kind permission from ASA, CSSA, SSSA.

biologically available in surface waters following hydrolysis by phosphatase enzymes, and many species of cyanobacteria grow well on a wide range of organic P compounds, including inositol hexakisphosphate [23]. DeLaune *et al.* [24] showed that while P concentrations in runoff were highly dependent on STP for unfertilized soils ($R = 0.93$); the relationship was extremely poor after poultry litter was applied to the same plots ($R = 0.004$), indicating that the effect of STP was overwhelmed when poultry litter was surface applied. This study further demonstrated that annual P loads in runoff water were closely correlated with the soluble P concentration of poultry litter ($R = 0.87$) than to the total P content of the litter ($R = 0.67$). Despite the importance of organic P in the P transfer process, its precise role remains poorly understood. However, the fact is now accepted that diffuse P pollution cannot be effectively managed without a comprehensive appreciation of the role of organic P [21].

7.2
Consequences of P Overloading

Eutrophication is the enrichment of surface waters by plant nutrients, a form of pollution that restricts the potential use of impacted water bodies. Eutrophication of fresh waters is a growing environmental problem worldwide and excess P is well documented as its most common cause in many aquatic systems [14]. It decreases dissolved oxygen and concurrently increases biological oxygen demand of a water body, adversely affecting aquatic wildlife and species diversity. Consequences of eutrophication include increased biomass of phytoplankton; shifts in phytoplankton to bloom-forming species; increase in blooms of gelatinous zooplankton (in marine environments); increased biomass of benthic and epiphytic algae; changes in macrophyte species composition and biomass; death in coral reefs and loss of coral reef communities; decrease in water transparency, taste, and odor; increased incidence of fish kills; reductions in harvestable fish and shellfish; decrease in esthetic value of the water body; and so on [27].

Losses of phosphorus from agricultural soils in the United States have recently been identified as one of the major causes of decreased surface water quality [28]. Nonpoint sources are now the dominant inputs of P to most US surface waters (Table 7.3). The geographical concentration of the poultry and swine industry and its vertically integrated nature have caused water quality problem in many parts of the United States. For example, the City of Tulsa, OK, derives its municipal drinking water supplies from the Eucha/Spavinaw Watershed at Arkansas–Oklahoma boarder. This watershed has a high density of broiler houses. Excessive algal blooms in the two lakes have caused very high levels of geosmin [*trans*-1,10-dimethyle-*trans*-9-decalol] present in the drinking water in recent years [18]. Geosmin is an earthy smelling metabolite of several organisms including actinomycetes and cyanobacteria such as *Anabaena* [29]. Geosmin starts causing taste and odor problems in water supplies when levels exceed $25\,\mathrm{ng\,l^{-1}}$. Research on cyanobacterial toxins has advanced greatly since the mid-1980s, focusing on their characterization and

Table 7.3 Phosphorus discharge to surface waters from nonpoint and point sources in the United States.

Source	Phosphorus (10^3 mg per year)
Croplands	615
Rangelands	242
Forests	495
Pastures	95
Other rural lands	170
Other nonpoint sources	68
Total nonpoint sources	1658
Total point sources	330
Nonpoint as percentage of total	84%

Source: Adapted from Ref. [27].

distribution [30, 31]. Toxic strains are now recognized as having a wide distribution throughout lakes and have been divided into the fast acting neurotoxins and the slower acting hepatotoxins. For example, 65 different types of the commonly occurring microcystin toxin have been identified and these are not simply confined to the genus *Mcrocystis*. Impacts of toxin vary widely; Anatoxin A(s), for an example, is an organophosphorus compound that acts as a cholinesterase inhibitor; it may not thus be logical to say that P in the aquatic environment is nontoxic [31]. Some of these are tumor promoters and a study from China links eutrophic drinking water sources to an elevated incidence of liver cancer in humans [32]. One potentially carcinogenic agent is trihalomethanes (THM) that can enter potable water supplies if – during chlorination process – chlorine reacts with dissolved organic compounds formed by algal cell lysis or algal excretion. The most tragic manifestation of the impact of toxins was seen in 1997, when 55 persons died within 7 months of exposure to cyanobacterial toxins in Brazil [33]. Reports from Australia, along with the Brazil episode, of adverse effects on human health associated with eutrophic drinking water – contaminated with cyanobacterial toxins – have led to an increased awareness of toxins as an environmental health issue [31]. In addition to toxins, cyanobacteria, and also algae, can produce other dissolved organic compounds, principally geosmin and isoborneol, which cause taste and odor problems [34].

The nature, degree, and implications of exposure to algal toxins via potable drinking water remain uncertain. However, phosphorus runoff from poultry and swine farms has also been implicated in the emergence of a dinoflagellate, *Pfiesteria piscicida*, in waterways on the eastern coast of the United States [35]. *P. piscicida* is a microscopic alga that lives in estuaries – where freshwater streams or rivers mix with salt water – along the Atlantic and Gulf coasts. Researchers at North Carolina State University first identified *P. piscicida* in 1988 in fish cultures. Since then, scientists have advanced many theories about the organism's life cycle and its possible effects on the health of fish and humans. In 1998, the US Congress appropriated funds to the Centers for Disease Control and Prevention (CDC) to address concerns about human health effects possibly associated with exposure to *P. piscicida*. Toxins from *Pfiesteria*

strains were isolated and characterized recently [35]. Eutrophication of the Chesapeake Bay, its tributaries, and other surface water systems in the Atlantic Coastal Plain continues to be a serious environmental problem [36].

7.3
Remediation Strategies

7.3.1
Chemical Amendments of Animal Waste or Manure

Methods to reduce the off-site movement of P from fields receiving manure have attracted much attention in recent times. As most of the P runoff from fields fertilized with poultry manure was in the dissolved form, several studies were conducted to determine if Al, Ca, and Fe amendments to poultry litter or manure could cause reduction in soluble P levels [37–39]. These studies demonstrated that chemical amendments of litter decreased soluble P levels by several orders of magnitude, and Al amendments particularly was better in controlling P losses than Ca amendments. It was also suggested that alum additions to poultry litter decrease P solubility by either direct precipitation of amorphous aluminum phosphate or by adsorption of P by aluminum hydroxide formed after the hydrolysis of alum takes place. Moor *et al.* [38, 39] measured P runoff from paired watershed, one of which was fertilized with alum-treated litter; the other with normal litter. They found that P concentrations in runoff were 73% lower in pastures fertilized with alum-treated poultry litter than the normal litter. Long-term studies on the effects of various fertilizers have also shown that soluble P levels in soils are much lower with alum-treated litter than untreated litter. Dou *et al.* [40] have shown the efficacy of different coal combustion by-products in stabilizing manure phosphorus. Three coal combustion by-products, namely, fluidized bed combustion (FBC) fly ash, flue gas desulfurization (FGD) by-product, and anthracite refuse fly ash (ANT), were added to dairy, swine, and broiler litter manures in a laboratory incubation study. FBC reduced readily soluble P by 50–60% at a rate of 400 g kg^{-1} for all three manures. FGD reduced soluble P further to nearly 80% when added to swine manure and broiler litter at 150 and 250 g kg^{-1}, respectively. ANT was not found effective for any of the manures tested. In all cases, reduction in readily soluble P was primarily associated with inorganic P (Pi) with little change in organic phosphorus (Po). In another study, efficacy of alum-amended poultry litter in reducing P release from three Delaware Coastal Plain soils, Evesboro loamy sand, Rumford loamy sand, and Pocomoke sandy loam, was investigated [41]. All soil types were incubated with alum-amended or unamended poultry litter. Long-term desorption of the incubated material resulted in approximately 12–13% reductions in cumulative P desorbed when comparing soil treated with unamended and alum-amended litter. In addition, the P release from the soil treated with alum-amended litter was not significantly different from the control (soil alone). The overall implication from this study is that the application of alum as a manure amendment is useful in coarse-textured soils of the Coastal Plain [41]. In one recent study, Wilson

et al. [42] showed that the application of mineral amendments to manure reduces P availability in manure (liquid dairy, laying hen, and broiler chicken) and soil without affecting crop productivity. Mehlich 3 extractable P (M3P) was reduced by 59–97% in all manure treated with mineral aluminum sulfate and ferric chloride. A container experiment was then carried out to examine the effect of soil with pretreated manure on timothy (*Phelum pretense* L.) growth and soil P levels [42]. Dairy manure plus aluminum sulfate (200 g kg^{-1}) reduced water-soluble P by 82% relative to the N–P–K control. This study demonstrated that mineral pretreatment of manure can reduce the soluble P content of the manure and soil without negative effects on plant growth.

The impact of alum addition on organic P transformations in poultry litter and litter-amended soil was investigated by Warren *et al*. [43]. Liquid-state ^{31}P nuclear magnetic resonance (NMR) indicated that phytic acid was the only organic P compound present, accounting for 50 and 45% of the total P in untreated and alum-treated litters, respectively, before incubation. After 93 days of storage-simulating incubation, phytic acid values declined to 9 and 37% in untreated and treated litters, respectively. Thus, these results show that adding alum to litter inhibits organic P mineralization during storage and promotes the formation of alkaline extractable organic P that sustains lower P solubility in the soil environment. The P speciation in poultry litter was analyzed using solid-state ^{31}P-NMR spectroscopy, and the mineralogy was analyzed by powder X-ray diffraction (XRD) after storing the samples moist and dried for up to 5 year under controlled conditions [44]. Struvite (magnesium ammonium phosphate mineral) concentrations were generally lower in dried samples than in samples stored moist. The moist samples also had higher concentrations of phosphate bound to aluminum hydroxides.

7.3.2 Chemical Amendments of Soil

Application of chemical amendments such as aluminum, calcium (Ca), and iron to soils enriched with P has also been tried in many studies [45–47]. The ability of water treatment residuals (WTR) to alter P solubility and leaching in Immokalee sandy soil amended with biosolids and triple superphosphate (TSP) was investigated in laboratory and greenhouse studies [46]. Several by-products are generated in drinking water purification process, but two major types of WTR are produced in large quantities and have potential for P immobilization. Conventionally, sedimentation–flocculation process produces residuals using either Al salts (Al-WTR) or Fe salts (Fe-WTR) as the primary coagulant. The other major residual type, Ca-WTR, is produced in water-softening facilities where lime is used for hardness removal. In above equilibration studies [46], the ability to reduce soluble P followed the order Al-WTR $>$ Ca-WTR $=$ Fe-WTR. Differences in the P-fixing capacity of the sesquioxide-dominated materials (Al-WTR and Fe-WTR) were attributed to their varying reactive Fe- and Al-hydrous oxide contents as measured by oxalate extraction. Leachate P was monitored from greenhouse columns where Bahia grass (*Paspalum notatum*) was grown on Immokalee soil amended with biosolids or TSP at an equivalent rate of 224 kg P ha^{-1} and WTR at 2.5% [46]. In the absence of WTR,

21% of TSP and 11% of biosolids total P leached over 4 months. With coapplied WTR, losses from TSP columns were reduced to 3.5% (Fe-WTR), 2–5% (Ca-WTR), and <1% (Al-WTR) of applied P. However, for biosolid treatments, all WTRs retarded downward P flux such that leachate P was not significantly different from control columns. In another growth chamber study [45], three different types of soil with M3P levels above 800 mg kg^{-1} were treated with 0–50 g kg^{-1} IRR (Fe-rich residue). Three crops of wheat were grown in succession. Water-soluble P and M3P concentrations were lowered with an increase in IRR application rate for each soil and correlated positively with tissue P for three soils. Biomass yield and tissue P concentrations were significantly reduced with the addition of IRR in all soil types [45]. Novak and Watts [47] also demonstrated that alum-based WTR can reduce extractable P concentrations in three P-enriched coastal plain soils. Incorporation of WTR into the three soils caused a near-linear and significant reduction in soil M3P and WSP concentrations (Figure 7.4). In two soils, 6% WTR [w/w] application caused a soil M3P concentration decrease to below the soil P threshold level (150 mg kg^{-1} soil). The results also showed that WTR incorporation into soils with higher P concentrations caused larger relative reductions in extractable WSP than M3P concentrations [47].

Chemical amendments do not prevent the accumulation of P in soils but merely reduce the amount of water-soluble phosphorus, thus regulating the runoff loss [48]. Moreover, P immobilization in soil by these amendments may not be stable on a long-term basis and instead result in higher soluble phosphates as in the case of Ca and ferric phosphate dissolution under certain normal soil conditions [49]. Though the use of Al salts to precipitate P in manures or soils is considered a better choice [49], these applications may also affect soil chemistry on a long-term basis. The stability of the P complexes formed with Al-oxides, as it relates to P lability in the environment, is uncertain [41].

7.3.3
Animal Diet Modification with Phytase

One of the major breakthroughs in recent years has been the commercialization of the phytase enzyme for use in swine and poultry feeds and its positive impact on P excretion. Phytase mediates the conversion of phytate – an organic form of P – into bioavailable P in diet, thus decreasing the need of feed P quantity [50]. The use of diet modifications to decrease the total P concentration in poultry and swine manure, and hence decrease P loading rates on agricultural lands and P transfer to surface waters, is considered an effective way of source management. Incorporating present technologies into diet formulations could reduce the amount of P in poultry manures and litters by at least 40%, while it is anticipated that future technologies could lead to decreases of more than 60% [50]. However, concerns were raised that although phytase can decrease total P in litter, it could increase the water-soluble phosphorus in the litter and hence the potential for P losses to surface waters following land application [51]. DeLaune *et al.* [24] found the average P concentration in the runoff from litter of birds fed phytase was 85 mg P l^{-1}, compared to 39 mg P l^{-1} from the litter of birds fed the normal diet, even though total P application rate used in their

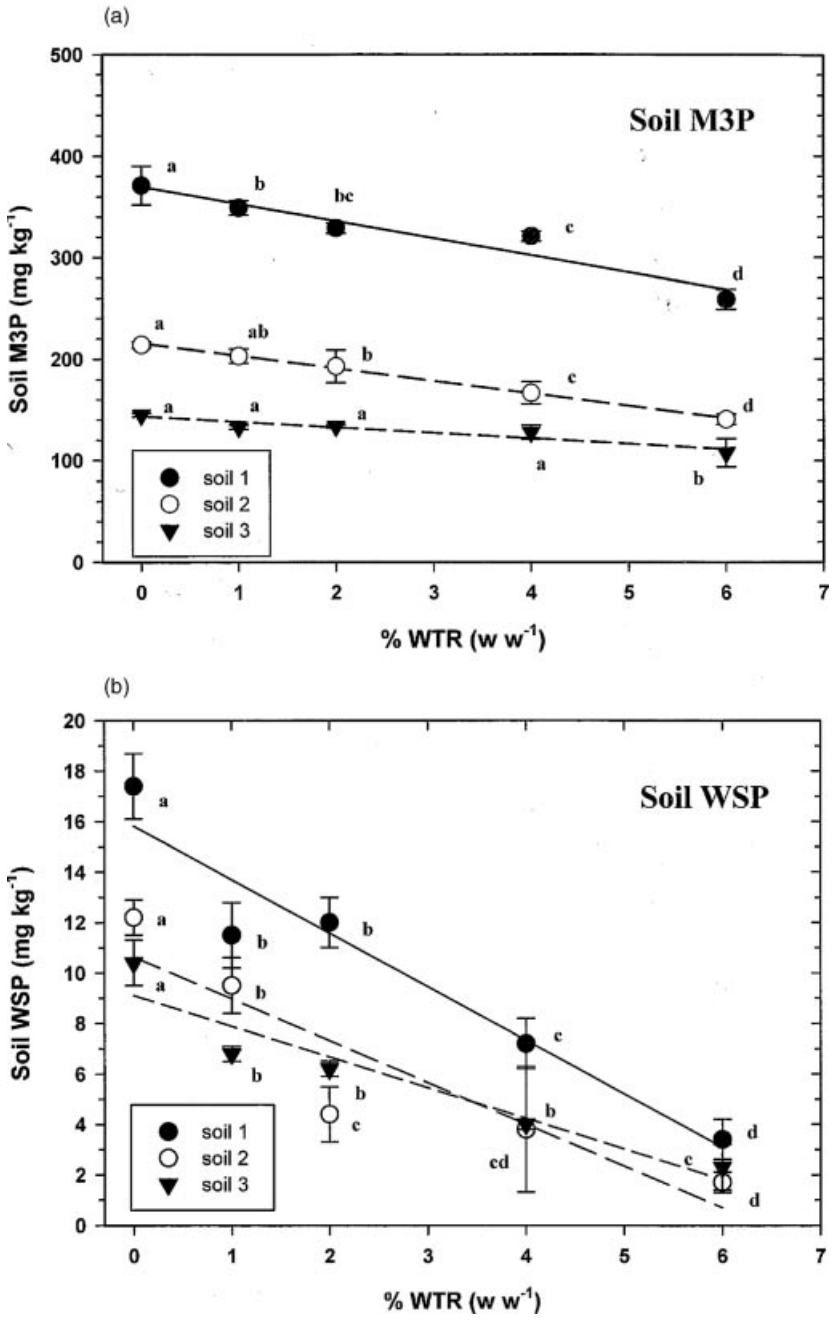

Figure 7.4 Linear regression relationship between reductions in soil Mehlich 3 phosphorus (a) and water-soluble (WSP) concentrations (b) and percent water treatment residual (WTR) incorporation. Reproduced with kind permission from the Journal of Environmental Quality [47].

experiment was roughly 40% lower with the phytase diet than normal diet. These researchers showed that the increased P concentration in runoff water was due to higher soluble P in the phytase litter than normal litter (3283 versus 1176 mg P kg^{-1}). Studies by Maguire *et al.* [52, 53] and Smith *et al.* [54], however, contradict previous findings and suggest that dietary phytase actually decreases both litter water-soluble P and dissolved P in runoff following litter application. Like poultry feed, swine diets were also amended with phytase in a number of studies. These studies conclusively show that digestibility of (or bioavailability) of P in cereal grains and oilseed meals is markedly improved with phytase supplementation. This means that reduced amounts of supplemental inorganic P are needed in swine diets to maximize growth and bone mineralization. As a result, fecal P is markedly reduced when phytase is included in the diet. Again, the fear of increased soluble P in swine litter following phytase treatment was allayed by several studies [54, 55]. Smith *et al.* [54] countered that dietary phytase significantly decreased water-soluble P in manure by 17%, but had no significant effect on dissolved P loss in runoff. However, a study at the University of Kentucky by Xavier *et al.* [56] presented a mixed picture showing 41% reduction in total P excretion, >2.5% increase in soluble P, and an insignificant difference in the amount of soluble P per day in swine excreta when phytase-supplemented diets were compared to control diets. Abioye *et al.* [57] conducted a study to determine if higher phytase levels can result in greater reduction in swine manure P without increased P solubility. These researchers reported that the greatest reduction in manure P was in pigs fed a diet containing 2000 U phytase per kg (without supplemental P), with 33% reduction in manure P. Although, there has been a great deal of research on the efficacy of phytase and other dietary strategies in poultry and swine performance and P excretion, little data exist on how these technologies will affect P runoff from fields fertilized with animal manure.

7.3.4
Phytoremediation

Various methods have been attempted to reduce soil P and halt the loss of P via runoff and leaching. These strategies though produce a varying degree of results, none seems to work without environmental risks. Alternatively, plant-assisted extraction of phosphate (Pi) could be an attractive strategy. Current P uptake rates are low for common row crops and forage grasses used to assimilate P from soil [58]. It is felt that the present cropping systems will require several decades, at the normal rate of P removal by plants, to reduce high P concentrations to an environmentally safe level. Thus, it is important to develop a method for the rapid removal of soil phosphorus. Mining of soil P, which includes harvesting P taken up from the soil by a crop grown without external P application, has been proposed as a possible management strategy for P-enriched soils [5, 7, 59, 60]. Phytoremediation is an inexpensive, nonintrusive, and often highly effective technique [61]. Plant-based cleanup strategies offer a number of advantages both over traditional cleanup methods and over other bioremediation technologies. There are several reports of metal hyperaccumulators

that are immensely useful in phytoremediation [61, 62]. Plants, generally referred to as metal hyperaccumulators, have the inherent potential to survive and accumulate excessive amounts of metal ions in their biomass without incurring damage to basic metabolic functions [61]. However, the ability of vegetation to assist in the remediation of P remains largely unknown. Some researchers suggest that for P phytoremediation to be effective, plants should have high biomass and accumulate P significantly higher ($\geq$1% DW) than the common plants do [7]. P remediation potentials of a number of crops were evaluated in a pot and field study indicating a differential pattern of phosphate (Pi) uptake by those crops [63]. Other studies also indicate usefulness of phytoremediation using stargrass [60] and perennial ryegrass [5] for P-impacted soils. Studies reflect the usefulness of plant-assisted P remediation, but no phosphorus hyperaccumulator has been identified [5, 60]. Both soil and crop management practices may thus require optimization for the P hyperaccumulator plant to compete with other plant species.

7.3.4.1 P Accumulation Potential of Annual Ryegrass

7.3.4.1.1 Hydroponic Study Annual ryegrass (*Lolium multiflorum*) is a closely related and interfertile species with perennial ryegrass (*L. perenne*), and both are grown all over the world as key forage grasses [64]. These are among the most palatable and highly digestible grasses for livestock. Annual ryegrasses, screened among several grass species for tolerance to high P levels, were selected for tissue P accumulation study [65]. A hydroponic method was preferred in this study to eliminate the effects of variable soil pH and microflora on P solubility and mobility. The objectives primarily were to (i) determine Pi accumulation potentials in Marshall and Gulf ryegrass, two cultivars of *L. multiflorum*, grown in a nutrient medium enriched with high levels of Pi, and (ii) to map out the Pi transport pathways across plant tissues. Both grasses accumulated high concentrations of P (>2% of tissue dry weight) in their roots and shoots in a medium containing 5 g l^{-1} KH_2PO_4 (Figure 7.5). Root P declined while shoot P rose rapidly with further increase in Pi in the medium.

The channel of P transport and accumulation in leaves and roots of ryegrass was mapped out by scanning electron microscopy (SEM) and electron dispersive X-ray spectroscopy (EDS) techniques. The SEM of roots excised from experimental plants (grown on 5 g l^{-1} KH_2PO_4) showed a higher accumulation of P in epidermal and cortical cells. Bright fibrillar spots of Pi can be seen in these cells (Figure 7.6a). These observations support the notion that epidermal and cortical cells are the primary entry points for P uptake. Though cells of the stellar region show intense formation of bright patches, P is distributed unevenly throughout the root section. In the micrograph of control roots (supplied with no Pi), such bright formations are conspicuously absent (Figure 7.6b). Leaf samples from the experimental plants showed concentrations of brighter P spots from pericycle to epidermis. Some of the epidermal and cortical cells revealed the presence of bright fluffy structures rich in P and K (Figure 7.6c). SEM of control leaf samples is clearly distinguishable from those of P-supplemented plant specimen with respect to fluffy structures (Figure 7.6d).

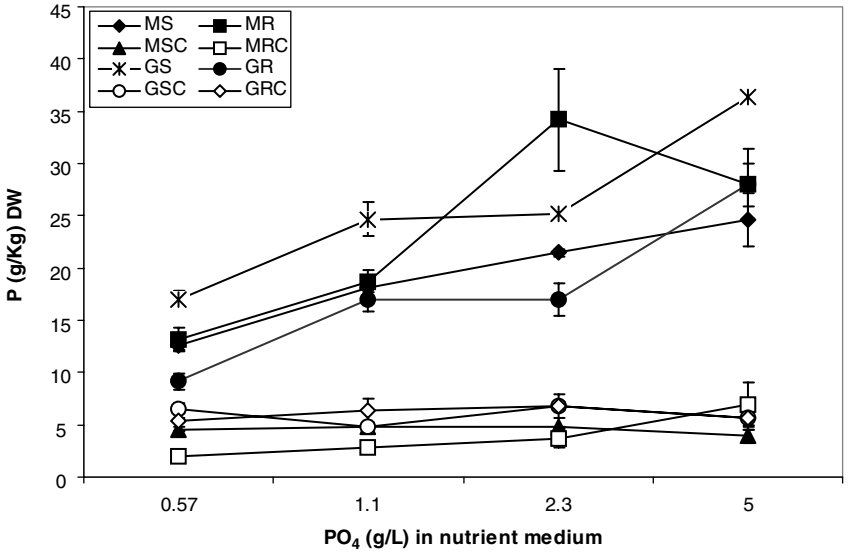

Figure 7.5 Accumulation of P in Marshall and Gulf ryegrass grown in nutrient solution containing 0.57–5 g l^{-1} of KH_2PO_4 for 2 weeks. Corresponding controls were set up with equal amounts of K_2SO_4. Each point represents a mean of six replicates (± SE). MS: Marshall grass shoot; MSC: Marshall grass shoot control; GS: Gulf grass shoot; GSC: Gulf grass shoot control; MR: Marshall grass root; MRC: Marshall grass root control; GR: Gulf grass root; GRC: Gulf grass root control. Reprinted from [65] with permission from American Chemical Society, © 2004.

The EDS analysis (P peaks) of KH_2PO_4-exposed plant parts is also distinguishable from those of controls (Figure 7.7a–d). SEM and EDS observations presented in this study lead to the belief that high levels of Pi flow into the cells via symplastic (via cell-to-cell connections) pathways. However, apoplastic (outward flow via cell walls) migration of Pi cannot be ruled out. Both apoplastic migration and symplastic transportation of heavy metals have been reported in heavy metal hyperaccumulator species [66].

7.3.4.1.2 **Greenhouse Study** As annual ryegrass demonstrated a large P accumulation in hydroponic conditions [65], experiments were designed to characterize phosphate accumulation from P-impacted soils under greenhouse conditions [67]. Biomass of plants increased with increasing concentrations of soil P until the concentration reached to a level of 20 g P kg^{-1} soil, where growth was affected. Significant increase ($p < 0.05$) in biomass with respect to control and also among the treatments was observed in both grass types supplied with P up to 10 g kg^{-1} soil, while decrease in biomass was significant ($p < 0.05$) at 20 g P kg^{-1} soil. Both crops accumulated increasing amounts of P ($p < 0.05$) in their shoots and roots with an increase in soil P (Figure 7.8a and b). P accumulations in Gulf ryegrass varied from 8.2 to 13 g kg^{-1} shoot dry weight, while P accumulations in Marshall ryegrass was 7.8–11 g kg^{-1} shoot dry weight depending on soil P concentrations [67].

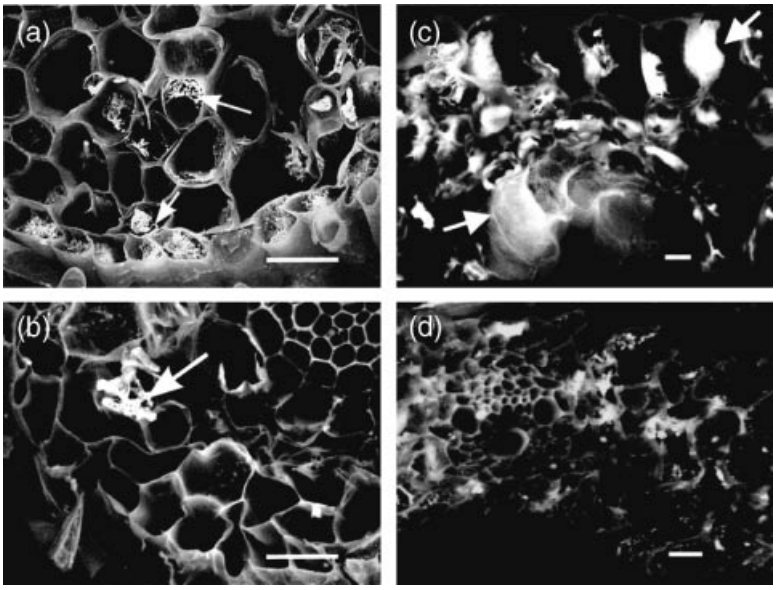

Figure 7.6 Scanning electron micrographs of the root of Gulf ryegrass seedlings grown in modified Hoagland's medium in the presence (a) and absence (b) of 5 g l^{-1} of KH_2PO_4. (a) Root section (scale marker = 10 μm) shows bright fibrillar spots (arrowheads) in epidermal and cortical cells. (b) Control root section (scale marker = 10 μm) shows no comparable spots (arrowhead indicates a dense, superimposed sheet of aluminum as a contaminant). Scanning micrographs of the leaf of Marshall ryegrass seedlings grown in modified Hoagland's medium in the presence (c) and in the absence (d) of 5 g l^{-1} of KH_2PO_4. (c) Leaf section (scale marker = 10 μm) shows abundance of bright fluffy structures (arrowheads) in epidermal and cortical cells. (d) Control leaf section (scale marker = 10 μm) shows no such structures. Reprinted from [65] with permission from American Chemical Society, © 2004.

In another study, three cool-season turf grasses, Kentucky bluegrass (*Poa pratensis*), tall fescue (*Festuca arundinaceae*), and perennial ryegrass (*L. perenne*), were investigated for phosphate removal capacity from enriched soils [68]. Shoot P differed significantly among these three grasses ranging from 0.3 to 0.45% of dry mass. This study also showed that genetic differences in P absorption might exist among turf grasses at both the interspecific and intraspecific levels.

Effect of pH on P Accumulation The form of P most readily accessed by plants is orthophosphates (Pi) and their forms in soil solution change according to soil pH [69]. The p*K* values for the dissociation of H_3PO_4 into $H_2PO_4^-$ and then into HPO_4^- are 2.1 and 7.2, respectively. Thus, below pH 6.0, most Pi will be present as the monovalent $H_2PO_4^-$ species, whereas H_3PO_4 and HPO_4^- will be available only in trace amounts [69]. Plant uptake is also affected by fixation of P by soil components, which is greatest in the presence of Fe- and Al-hydroxylated surfaces and, at higher pH, calcium carbonate [70]. Therefore, to study how varying soil pH conditions in the Pembroke silt loam influence P uptake in Gulf and Marshall ryegrass, plants were grown in P-enriched (2.5 g P kg^{-1} soil) soils maintained at pH 5.6, 6.5, and 7.8 [67]. A significant increase in shoot P was observed in both grass types at pH 5.6 with respect to

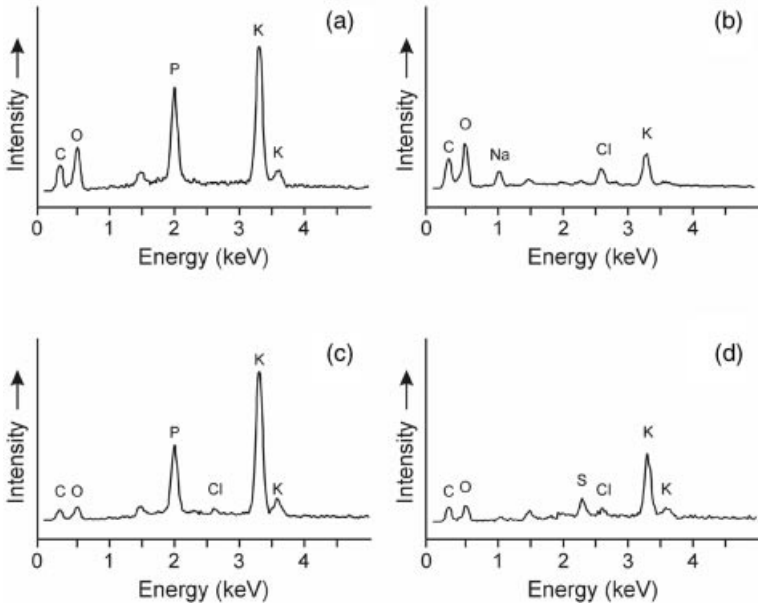

Figure 7.7 Electron dispersal X- ray spectroscopic analysis. (a) Spectrum of one of the bright spots (arrowheads) shown in Figure 5a. (b) Spectrum of control root shown in Figure 5b. (c) Spectrum of one of the bright fluffy structures (arrowheads) shown in Figure 5c. (d) Spectrum of control leaf shown in Figure 5d. Reprinted from [65] with permission from American Chemical Society, © 2004.

accumulation at pH 7.8 (Figure 7.9a and b). However, difference in shoot P between pH 6.5 and 7.8 was significant ($p < 0.05$) in Gulf but not in Marshall ryegrass. Most studies on the pH dependence of Pi uptake in higher plants have found that uptake rates are highest between pH 5.0 and 6.0, where plant-assimilable $H_2PO_4^-$ dominates [69].

The experiment was also designed to study the effect of the changing temperature that may be encountered by the crops during different seasons on the dry mass productivity and corresponding P uptake [67]. Variations in the shoot dry matter and P accumulations were significant ($p < 0.05$) at different temperature regimes in these grasses (Table 7.4). Biomass growth in Marshall ryegrass was greater than Gulf ryegrass at all temperatures, which is consistent with earlier studies involving solution culture [65]. As differences in biomass growth are greater, total P removal capacity of these plants will also be significantly different. Studies suggest that air or soil temperature may influence both dry mass accumulation and P uptake in plants [71, 72]. Cool soil temperatures generally result in reduced P uptake from soil reserves by plant roots. Even soils with high levels of P may not provide adequate P to plants when the temperature is suboptimal during the cold season. Annual ryegrass is generally cultivated as winter crop in the temperate climates, but this study suggests that they can be grown also during the summer when temperature exceeds 30 °C, while serving the purpose of P mining.

Production of phosphatases is a potentially important way for plants to enhance P availability, as a large proportion of soil P occurs in organic forms [73]. Phosphatases are required for the mineralization of organic forms of soil P to release phosphate (Pi) – the form of P readily acquired by plants. More recently, phytase (EC 3.1.3.26), a class

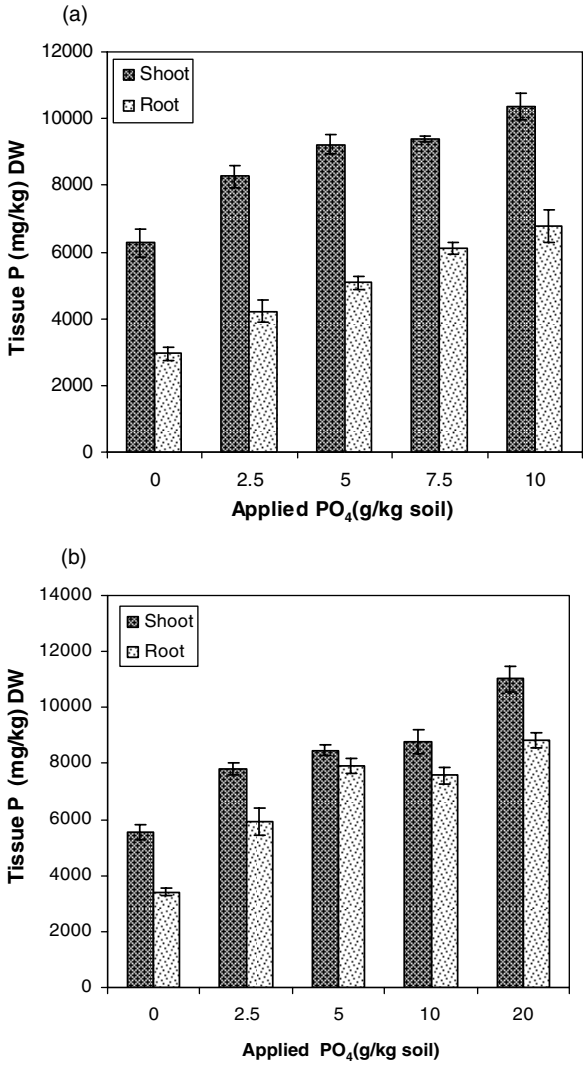

Figure 7.8 (a) P accumulation in Gulf ryegrass grown in soils enriched with 0–10 g P kg^{-1} soil for 6 weeks. (b) P accumulation in Marshall ryegrass grown in soils enriched with 0–20 g P kg^{-1} soil for 6 weeks. Values represent four replicates ± standard error of the mean. Reprinted from [67] with permission from American Chemical Society, © 2005.

of phosphomonoesterases with high specific activity against phytate, has been described in plant roots [73]. Acid phosphomonoesterase and phytase activities in the annual ryegrass plant roots – grown in acidic and slightly alkaline soils under P sufficiency or P deficiency conditions – were determined [67]. The results indicate that phosphomonoesterase and phytase activities were more or less similar in both Marshall and Gulf grasses when grown in acidic soils, but activities were significantly higher in Marshall than Gulf when grown in alkaline soil (Table 7.5).

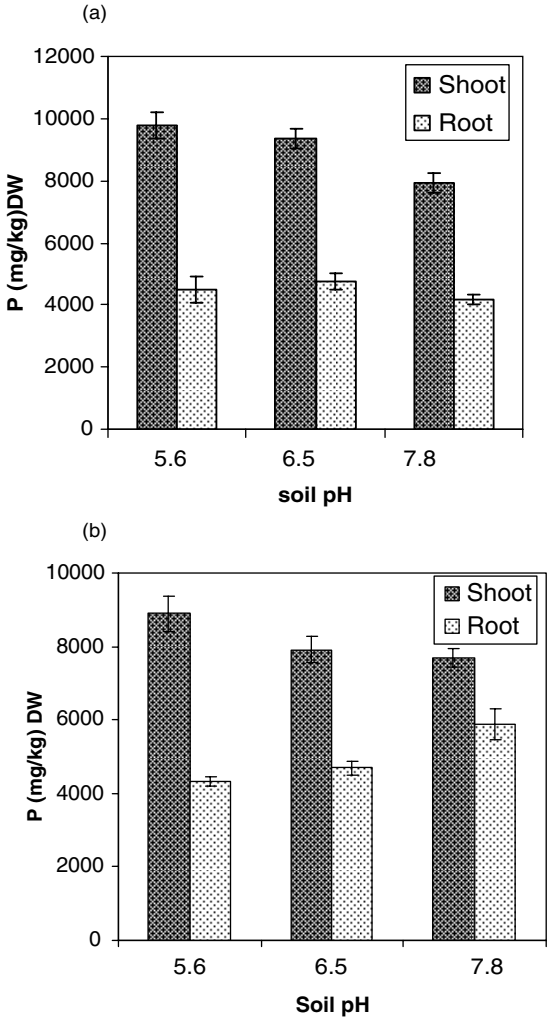

Figure 7.9 (a) P accumulation in Gulf ryegrass grown in soils (pH 5.6–7.8) enriched with 2.5 g P kg^{-1} soil for 6 weeks. (b) P accumulation in Marshall ryegrass grown in soils (pH 5.6–7.8) enriched with 2.5 g P kg^{-1} soil for 6 weeks. Values represent five replicates ± standard error of the mean. Reprinted from [67] with permission from American Chemical Society, © 2005.

Phosphomonoesterase activity in annual ryegrass was significantly higher [67] than the corresponding values reported for wheat grown in sterile medium containing various sources of P [74]. Phytase activity expressed in terms of a percentage of the total phosphomonoesterase activity was low (0.7–1.0%) in annual ryegrass but greater than *Arabidopsis* [75] and pasture grasses [76]. Plants with high phytase activity in their roots can hydrolyze phytates, which account for a large proportion of unavailable soil P pool, and can thus deplete excess P source more efficiently [75]. The enzyme activities in annual ryegrass also varied in respect of soil pH (Table 7.5). The activities

Table 7.4 Effect of temperature on shoot biomass and P accumulation in annual ryegrass grown in P-enriched soil*.

Treatment temperature (°C)	Biomass (g dry weight per pot)		P (mg kg^{-1} shoot dry weight)	
	Gulf	Marshall ryegrass	Gulf	Marshall ryegrass
20	0.84**	1.00[a]	7900[a]	7500[a]
24	1.11[b]	1.68[b]	8200[a]	7800[a]
28	1.38[c]	1.46[b]	9400[b]	8500[b]
32	0.60[a]	0.85[a]	9100[c]	8300[b]

Source: Sharma and Sahi [67].
*P was applied at the rate of 2.5 g kg^{-1} soil.
**Values are the mean of three replicates and, within each column, those not followed by the same letter are significantly different ($p < 0.05$).

were about twofold higher in alkaline soil than acidic ones in case of both enzyme types. Possibility of P immobilization with Ca under alkaline condition may necessitate conditions for plants to express high enzyme activity [67].

The effect of P supply on the activity of enzymes was also significant in annual ryegrass where both phosphomonoesterase and phytase activities were higher in P-rich plants than P-deficient plants [67]. This feature, though not uncommon, was not compatible with many studies that show enhanced activities, particularly of phytase in plant root extracts [76, 77]. Therefore, enhanced P uptake in annual ryegrass though cannot be directly correlated with the determined enzyme activities, the interesting pattern in enzyme activities may be one of the unique features that influence P nutrition and accumulation in these plants [67].

Table 7.5 Acid phosphomonoesterase and phytase activities of root extracts in annual ryegrass grown in P-enriched[a)] acidic and slightly alkaline soils for 5 weeks.

Treatments	Acid phosphomonoesterase activity mU g^{-1} root FW	Phytase activity mU g^{-1} root FW
Acidic soil (pH 5.7)		
Gulf ryegrass control (P^-)	371 ± 19.6[b)]	2.5 ± 0.62
Gulf ryegrass (P^+)	460 ± 38.7	3.2 ± 0.85
Marshall ryegrass control (P^-)	397 ± 14.1	2.3 ± 0.81
Marshall ryegrass (P^+)	431 ± 53.2	3.0 ± 0.33
Alkaline soil (pH 7.8)		
Gulf ryegrass control (P^-)	549 ± 83.3	3.6 ± 0.32
Gulf ryegrass (P^+)	722 ± 87.5	7.6 ± 1.01
Marshall ryegrass control (P^-)	693 ± 59.3	5.8 ± 0.91
Marshall ryegrass (P^+)	883 ± 46.6	7.0 ± 1.14

a) P was applied at the rate of 2.5 g kg^{-1} soil.
b) Values are the mean of three replicates ± standard error of the mean.
Source: Sharma and Sahi [67], reprinted with permission from American Chemical Society, © 2005.

7.3.4.2 Phytoremediation Potential of Food Plants

In an attempt to identify a P hyperaccumulator, several plants belonging to legume, vegetable, and herb (foliage) crops were screened in P-enriched soils in the greenhouse [78]. The goal was to identify economically valuable crop species that contain both high biomass and high shoot P. The promising plant species were further characterized for P accumulations under different soil P concentrations.

Among the various groups of plants (legumes, vegetables, and herbs), a large variation in P accumulation was noticed, and only few plant species demonstrated the P accumulation at a level of 1% (10 g P kg^{-1} shoot DW) or more in their shoots (Table 7.6). However, among herbs, pigweed (*Amaranthus* spp.), and goosefoot (*Chenopodium* spp.) plants were promising P accumulators, with some of the varieties accumulating in the range of 1.1–1.4% P (DW) in their leaves. The ratio of shoot-to-root P was also high (2 or >2) in these varieties. On the basis of P contents in aerial parts, these plants can be suitable for P phytoremediation, but some of the pigweed and goosefoot species are known for their invasive nature, and thus can be difficult to remove when not desired. However, a cautious approach involving harvesting shoots before flowering can be effective in reducing soil P levels, particularly, in fallow lands [78].

While screening vegetable crops, cucurbits, namely, cucumber and yellow squash, demonstrated an appreciable P accumulation in their aerial parts [78], consistent with earlier observations on their trials in the phosphatic clay soil [79]. With an accumulation of about 1.8% in stem and 1% in leaves (DW), cucumber can remove a substantial quantity of soil P. Like cucumber, yellow squash also accumulated over 1% (as much as 1.4% DW) in their aerial parts. Cucumber stem showed an increasing accumulation with increase in soil P concentration after 8 weeks, but leaves had higher accumulations depending on soil P after 12 weeks. It is likely that cucumber stems first acquire P from soil and then distribute it in parts like leaves and fruits after a period of time. Accumulation in fruits also increased significantly ($p < 0.05$) with time. The pattern of P accumulation in squash was different in that stems and leaves both had increasing concentrations of P as time increased. However, squash fruits had an accumulation of about 1.4% (DW), irrespective of P concentrations in soil. Even control plants grown in soils without any external addition of P had high P concentrations in fruits (after 8 weeks) and stems (after 12 weeks). This may be because of high efficiency of squash plants for P acquisition from soils having even low concentrations of P [78].

In an earlier study, shoot accumulations of P were determined for oil crops such as Indian mustard, canola, and rape oilseed, and a maximum level of 0.46% P (shoot DW) accumulation was observed in Indian mustard [63]. It was interesting to determine the P accumulation pattern in sunflower, which is one of the most widely used edible oil crops worldwide [78]. Sharma *et al.* [78] showed that sunflower accumulates more P than Indian mustard, canola, and rape oilseed with stem, leaf, and flower accumulations of 0.6, 0.8, and 1.0% (DW), respectively. The effect of soil P concentration (>1 g kg^{-1} soil) and duration of time had no significant effect on the accumulation potential of sunflower plants. Increase in root P was noticeable with increase in duration of time. Like cucumber and squash, sunflower also displayed growth in biomass in the presence of a high concentration of soil P as much as control

Table 7.6 Phosphorus content in shoots (stem and leaves) and roots of plants evaluated for phytoremediation potential in the greenhouse.

Plant	Shoot	Root	Shoot/Root
	[a)]P mg/kg dry weight (±S.E.)		P
Beans			
Kentucky wonder bean	6221 ± 549	6927 ± 255	0.8
Lima bean	3752 ± 299	5156 ± 427	0.7
Blue lake bean	4963 ± 370	4425 ± 421	1.1
Black-eyed pea	3324 ± 632	5096 ± 296	0.6
Pinto bean	3826 ± 264	5343 ± 700	0.7
Tender green	3362 ± 197	4291 ± 222	0.7
Royal burgundy	4361 ± 293	8012 ± 563	0.5
Bush wax	4145 ± 366	8073 ± 830	0.5
Chickpea	5111 ± 343	6876 ± 190	0.7
Moong bean	4992 ± 229	4741 ± 330	1.0
Kidney bean	4012 ± 310	4693 ± 531	0.8
Peas	3643 ± 167	4597 ± 442	0.7
Soybean	3912 ± 220	4312 ± 435	0.9
Lentil	3990 ± 288	3856 ± 638	1.0
Vegetable crops			
Cauliflower	5291 ± 542	9154 ± 883	0.5
Cabbage	5088 ± 660	8839 ± 592	0.5
Broccoli	3316 ± 310	3924 ± 186	0.8
Tomato	4217 ± 368	5946 ± 283	0.7
Egg plant	3812 ± 739	3712 ± 175	1.0
Bitter gourd	4612 ± 883	6123 ± 326	0.7
Sponge gourd	6730 ± 398	5892 ± 250	1.1
Star luffa	4625 ± 211	8723 ± 299	0.5
Edible morning glory	3812 ± 339	3612 ± 341	1.0
Carrot	4720 ± 429	5149 ± 333	0.9
Radish	5934 ± 200	6789 ± 257	0.8
Okra	4612 ± 364	6123 ± 332	0.7
Herbs			
Spinach	6547 ± 732	—	—
Lettuce	6628 ± 422	8723 ± 554	0.7
Parsley	4332 ± 328	4418 ± 281	0.9
English thyme	3297 ± 320	3460 ± 334	0.9
Cilantro	5433 ± 632	5585 ± 280	0.9
Dill	5889 ± 392	6486 ± 230	0.9
Basil	4322 ± 431	3853 ± 410	1.1

Source: Sharma *et al.* [78], reprinted with permission from Elsevier, © 2007.
Plants were grown in soils enriched with 2.5 g P kg^{-1} soil for 8 weeks.
a) Values are the mean of three replicates ± standard error of the mean.

plants. As this crop is known for its high biomass in fields, the cumulative P removal capacity may be significantly higher at the demonstrated levels of P accumulation in aerial parts. Besides element accumulation and harvestable biomass, what is important to determine the phytoextraction potential of a plant species is the depth of rooting zone, as suggested by Mertens *et al*. [80]. Notably, sunflower was observed to have extensive growth of root system extending deep into soils (data not presented) under high P conditions. That is also true of *Amaranthus* and *Chenopodium* species.

Sharma *et al*. [78] analyzed phosphomonoesterase and phytase activities in plant species that showed increased P accumulations (cucumber, squash, and sunflower). Activities of both enzymes were increased in cucumber and squash grown in P-enriched soils compared to the activities in controls (with no addition of P). When the activity of phosphomonoesterase increased to about 35% in both cucumber and squash, the phytase activity increased about 400% in cucumber and 40% in squash with respect to controls. Sunflower plants, however, demonstrated either comparable (phosphomonoesterase) or higher activity (phytase) under the P-deficient condition (controls). The pattern of enzyme activities in cucumber and squash was remarkably different from the pattern of activities in sunflower and other plant species [74, 76]. However, it was comparable to the enzyme activity in Marshall and Gulf ryegrass that also show higher P accumulations from P-enriched soils [67]. A similar trend was also observed in *Trifolium repens* (a legume pasture), which demonstrated higher phytase activity in high P conditions [76]. Plants having high phytase activity in their roots can hydrolyze phytates, which account for a large proportion of unavailable soil P pool, and can thus deplete excess P source more efficiently. Higher enzyme activities in cucumber and squash thus may be one of the factors contributing to their P accumulation efficiency. The molecular mechanism of P nutrition in plants under the conditions of P adequacy is not well known; however, much information is available on the acquisition of P under P deficiency [81]. Although the investigation by Sharma and Sahi [67] identified some economically important plant species with affinity for high P acquisition, the P accumulation potential of these plants needs to be verified under natural conditions of high soil P.

7.4 Conclusions

The present research strongly establishes the relationship between high-density animal production, often referred to as confined animal feeding operations (CAFOs), and high soil test P. Elevated STP again correlates directly with an accelerated P transfer to receiving lakes, streams, or watersheds. Increased P loading to aquatic ecosystems, and in particular lakes and rivers, is considered a prerequisite to eutrophication and water quality deterioration. All these associations have sparked global concerns, propelling the US Environmental Protection Agency to set up the National Regional Nutrient Criteria Program, European Commission to launch the Water Framework Directive, and other countries to find various water quality monitoring agencies, to identify water impairment and target remediation.

Remediation strategies ranging from chemical amendments of animal manures to soil applications of water treatment residues, to animal feed modifications, to the use of cover or buffer crops as scavengers of P have been tried to halt P transfer to aquatic ecosystems. Each method has its pros and cons. For example, chemical amendments of manure or soil were reported to decrease water-soluble or runoff P levels by several orders of magnitude, and Al amendment was particularly better in controlling P losses than Ca amendments. However, chemical amendments do not prevent the accumulation of P in soils but merely reduce the amount of water-soluble phosphorus, thus regulating the runoff loss. Moreover, P immobilization in soil by these amendments may not be stable on a long-term basis and instead may result in higher soluble phosphates as in the case of Ca and ferric phosphate dissolution under certain normal soil conditions. Animal feed modifications (particularly poultry and swine feed modifications) by addition of phytase enzyme show a great promise. Diet formulations based on present methodologies could reduce the amount of P in poultry manures and litters by 40%, while future technologies could lead to further P decreases, as anticipated by the US Council for Agricultural Science and Technology. However, this remedial measure is also not free from risks. Concerns were raised that although phytase can decrease total P in litter, it can increase the water-soluble phosphorus in the litter and hence the potential for P losses to surface waters following land application. In addition to finding an efficient P source management, phytoremediation could be another tier of output remediation as it involves application of P-accumulating plant species in scavenging excess P. Phytoremediation is a sustainable, relatively inexpensive, and highly effective technique. Harvesting P taken up from the soil by a crop grown without external P application has recently been argued as a possible management strategy for P-impacted soils. However, the ability of vegetation to assist in the remediation of P remains largely unknown. Studies suggest the usefulness of plant-assisted P remediation, but no phosphorus hyperaccumulator has been identified. The potential of phytoremediation can be fully realized when P hyperaccumulating plant types are identified and used in the process of P removal from soils. The P-accumulating species will find value enhancements also when applied as cover or buffer crops. As organic P is a critical contributor in P loadings to waterways, plant species capable of increased phytase secretion and organic P assimilation can be a desirable candidate in the search for a P accumulating plant. Recently constructed transgenic plant species that overexpress root phytase and accumulate P from organic P sources can be tested under field conditions for phytoremediation potential.

A variety of best management practices (BMPs) have recently been developed to minimize the potential for soil P transport. These include, but are not limited to, cultivation practice such as conservation tillage; the use of cover crops, for example, grasses, legumes, and other herbaceous species, as a seasonal cover to protect against soil erosion and P loss; methods of soil P testing such as STP or P index; artificial drainage, for example, tile drains; creation of buffers such as riparian buffers or filter strips, alley cropping, and vegetative barriers; and streambank protection. Research on all aspects of agricultural P, from new approaches to animal P nutrition, to modifying the physicochemical characteristics of P sources,

and to advancing our understanding of the interactions between soil and crop management on P transport, has intensified in the past decade. Research also shows that the use of BMP systems (combinations of several BMPs tailored to the specific conditions and environmental concerns at a given site) is a more effective approach of addressing P problems. The evolution of sustainable P management is an ongoing process that is the responsibility of all involved: from farmers to consuming public, to policy makers.

References

1 Tiessen, H. (2005) Phosphorus dynamics in tropical soils, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 253–262.

2 Fontes, M.P.F. and weed, S.B. (1996) *Geoderma*, **72**, 37–51.

3 Fairhurst, T., Lefroy, R., Mutert, E. *et al.* (1999) *Agrofor. Forum*, **9**, 2–8.

4 Tarkalson, D.D. and Mikkelsen, R.L. (2003) *Nutr. Cycl. Agroecosystems*, **66**, 295–303.

5 Koopmans, G.F., Chardon, W.J., Ehlert, P.A.I. *et al.* (2004) *J. Environ. Qual.*, **33**, 965–975.

6 Kellogg, R.L., Lander, C.H., Moffitt, D.C. *et al.* (2000) USDA-NRCS-ERS Pub. No. nps00-0579. Available on the internet at (www.nhq.nrcs.usda.gov/land/index/publication.html/) (accessed 1 May 2011).

7 Novak, J.M. and Chan, A.S.K. (2002) *Crit. Rev. Plant Sci.*, **21**, 493–509.

8 Coal, F.G. (2000) Phosphorus dynamics in soils of the chesapeake bay watershed: a primer, in *Agricultural Phosphorus in the Chesapeake Bay Watershed: Current Status and Future Trends* (ed. A.N. Sharpley), Chesapeake Research Consortium, Inc., Edgewater, MD, pp. 43–55.

9 Sims, J.T., Edwards, A.C., Schoumans, O.F. *et al.* (2000) *J. Environ. Qual.*, **29**, 60–71.

10 Maguire, R.O., Chardon, W.J., and Simard, R.R. (2005) Assessing potential environmental impacts of soil P by soil testing, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 145–180.

11 Cromwell, G.L. (2005) Phosphorus and swine nutrition, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 607–634.

12 Withers, P.J.A., Edwards, A.C., and Foy, R.H. (2001) *Soil Use Manage.*, **17**, 139–149.

13 Smaling, E.M.A., Oenema, O., and Fresco, L.O. (1999) Epilogue, in *Nutrient Disequilibria in Agrosystems: Concepts and Case Studies* (ed. E.M.A. Smaling *et al.*), CAB Int., Wallingford, Oxon, UK, pp. 295–313.

14 Sharpley, A.N., Daniel, T.C., Sims, J.T. *et al.* (2003) *Agricultural Phosphorus and Eutrophication*, 2nd edn, USDA-ARS, ARS-149, US Gov. Print. Office, Washington DC.

15 Foy, R.H. (2005) The return of the phosphorus paradigm: agricultural phosphorus and eutrophication, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 911–939.

16 Sims, J.T. and Kleinman, P.J.A. (2005) Managing agricultural phosphorus for environmental protection, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 1021–1068.

17 Hooda, P.S., Rendell, A.R., Edwards, A.C. *et al.* (2000) *J. Environ. Qual.*, **29**, 1166–1171.

18 Heathwaite, L., Sharpley, A., Bechmann, M. *et al.* (2005) Assessing the risk and magnitude of agricultural nonpoint source phosphorus pollution, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 981–1020.

19 Pierzynski, G.M., McDowell, R.W., and Sims, J.T. (2005) Chemistry, cycling and potential movement of inorganic phosphorus in soils, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 53–86.

20 Harrison, A.F. (1987) *Soil Organic Phosphorus: A Review of World Literature*, CAB Int., Wallingford, Oxon, UK.

21 Condron, L.M., Frossard, E., Newman, R.H. *et al.* (1997) Use of ^{31}P NMR in the study of soils and the environment, in *Nuclear Magnetic Resonance Spectroscopy in Environmental Chemistry* (ed. M.A. Nanny), Academic Press, New York, pp. 247–271.

22 Chardon, W.J., Oenema, O., del Castilho, P. *et al.* (1997) *J. Environ. Qual.*, **26**, 372–378.

23 Whitton, B.A., Grainger, S.L.J., Hawley, G.R.W. *et al.* (1991) *Microb. Ecol.*, **21**, 85–98.

24 DeLaune, P.B., Moor, P.A., Jr., Carmon, D.K. *et al.* (2004) *J. Environ. Qual.*, **33**, 2183–2191.

25 Condron, L.M., Turner, B.L., and Cade-Menun, B.J. (2005) Chemistry and dynamics of soil organic phosphorus, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 87–121.

26 Condron *et al.* (2005) Chemistry and dynamics of soil organic phosphorus, in *Phosphorus: Agriculture and the Environment* (eds J.T. Sims and A.N. Sharpley), ASA-CSSA-SSSA, Madison, Wisconsin, pp. 87–121.

27 Carpenter, S.R., Caraco, N.F., Correll, D.L. *et al.* (1998) *Ecol. Appl.*, **8**, 559–568.

28 US Environmental Protection Agency (2000) A Summary of the National Water Quality Inventory: 1998 Report to Congress. EPA841-S-00-001.

29 Izaguirre, G., Hwang, C.J., Krasner, S.W. *et al.* (1982) *Appl. Environ. Microbiol.*, **43**, 708–714.

30 Codd, G.A. (2000) *Ecol. Eng.*, **16**, 51–60.

31 Backer, L.C. (2002) *Lake Reserv. Manage.*, **18**, 20–31.

32 Yu, S.Z. (1995) *J. Gastroenterol. Hepatol.*, **10**, 674–682.

33 Jochimsen, E.M., Carmichael, W.W., An, J.S. *et al.* (1998) *New Engl. J. Med.*, **338**, 873–878.

34 Cooke, G.D. and Kennedy, R.H. (2001) *Lake Reserv. Manage.*, **17**, 157–174.

35 Burkholder, J.M., Gordon, A.S., Moeller, P.D. *et al.* (2005) *Proc. Natl. Acad. Sci. USA*, **102**, 3471–3476.

36 Butler, J.S. and Coal, F.J. (2005) *J. Environ. Qual.*, **34**, 371–381.

37 Moore, P.A., Jr. and Miller, D.M. (1994) *J. Environ. Qual.*, **23**, 325–330.

38 Moore, P.A., Jr., Daniel, T.C., and Edwards, D.R. (1999) *Poult. Sci.*, **78**, 692–698.

39 Moore, P.A., Jr., Daniel, T.C., and Edwards, D.R. (2000) *J. Environ. Qual.*, **29**, 37–49.

40 Dou, Z., Zhang, G.Y., Stout, W.L. *et al.* (2003) *J. Environ. Qual.*, **32**, 1490–1497.

41 Staats, K.E., Arai, Y., and Sparks, D.L. (2004) *J. Environ. Qual.*, **35**, 1904–1911.

42 Wilson, J.D., Zheljazkov, V.D., Rathgeber, B. *et al.* (2008) *Soil Sci. Plant Nutr.*, **54**, 600–605.

43 Warren, J.G., Penn, C.J., McGrath, J.M. *et al.* (2008) *J. Environ. Qual.*, **37**, 469–476

44 Hunger, S., Sims, J.T., and Sparks, D.L. (2008) *J. Environ. Qual.*, **37**, 1617–1625.

45 Codling, E.E., Mulchi, C.L., and Chaney, R.L. (2002) *Comm. Soil Sci. Plant Anal.*, **33**, 1063–1084.

46 Elliott, H.A., O'Connor, G.A., Lu, P. *et al.* (2002) *J. Environ. Qual.*, **31**, 1362–1369.

47 Novak, J.M. and Watts, D.W. (2005) *J. Environ. Qual.*, **34**, 1820–1827.

48 Hunger, S., Sims, J.T., and Sparks, D.L. (2005) *J. Environ. Qual.*, **34**, 382–389.

49 Smith, D.R., Moor, P.A., Jr., and Griffis, C.L. (2001) *J. Environ. Qual.*, **30**, 992–998.

50 Council for Agricultural Science and Technology (2002) Issue Paper 21, CAST, Ames, IA.

51 Vadas, P.A., Meisinger, J.J., Sikora, L.J. *et al.* (2004) *J. Environ. Qual.*, **33**, 1845–1854.

52 Maguire, R.O., Sims, J.T., and Applegate, T.J. (2005) *J. Environ. Qual.*, **34**, 359–369

53 Maguire, R.O., Dou, Z., Sims, J.T. *et al.* (2005) *J. Environ. Qual.*, **34**, 2093–2103.

54 Smith, D.R., Moor, P.A., Jr., Maxwell, C.V. *et al.* (2004) *J. Environ. Qual.*, **33**, 1048–1054.

55 Baxter, C.A., Joern, B.C., Ragland, D. *et al.* (2003) *J. Environ. Qual.*, **32**, 1481–1489.

56 Xavier, E.G., Pretty, L.A., Cromwell, G.L. *et al.* (2004) *J. Anim. Sci.*, **82** (Suppl 1), 253.

57 Abioye, S., Ige, D., Akinremi, O. *et al.* (2010) *J. Agri. Sci.*, **2**, 3–12.

58 Whitehead, D.C. (2000) *Nutrient Elements in Grasslands: Soil–Plant–Animal Relationship*, CABI Publishers, New York.

59 Frossard, E., Condron, L.M., Oberson, A. *et al.* (2000) *J. Environ. Qual.*, **29**, 15–23.

60 Pant, H.K., Mislevy, P., and Rechcigl, J.E. (2004) *Agron. J.*, **96**, 1299–1305.

61 Cunningham, S.D., Shan, J.R., Crowley, J.R. *et al.* (1997) *Phytoremediation of Soil and Water Contaminants* (eds E.L. Kruger, T.A. Anderson, and J.R. Coats), American Chemical Society, Washington, DC, pp. 2–17.

62 Blaylock, M.J., Salt, D.E., Dushenkov, S. *et al.* (1997) *Environ. Sci. Technol.*, **31**, 860–865.

63 Delorme, T.A., Angle, J.S., Coale, F.J. *et al.* (2000) *Inter. J. Phytorem.*, **2**, 173–181.

64 Jauhar, P.P. (1993) *Monographs on Theoretical and Applied Genetics* (eds R. Frankel, M. Grossman, and H.F. Linskens *et al.*), Springer, Berlin, **18**, pp. 243.

65 Sharma, N.C., Sahi, S.V., Jain, J.C. *et al.* (2004) *Environ. Sci. Technol.*, **38**, 2443–2448.

66 Sahi, S.V., Bryant, N.L., Sharma, N.C. *et al.* (2002) *Environ. Sci. Technol.*, **36**, 4676–4680.

67 Sharma, N.C. and Sahi, S.V. (2005) *Environ. Sci. Technol.*, **39**, 5475–5480.

68 Liu, H., Hull, R.J., and Duff, D.T. (1995) *J. Plant Nutr.*, **18**, 523–540.

69 Furihata, T., Suzuki, M., and Sakurai, H. (1992) *Plant Cell Physiol.*, **33**, 1151–1157.

70 Comerford, N.B. (1998) *Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic, and Ecosystem Processes* (eds J.P. Lynch and J. Deikman), American Society of Plant Physiology, Rockville, MD, USA, pp. 136–147.

71 Baon, J.B., and Smith, S.E., and Alston, A.M. (1994) *J. Plant Nutr.*, **17**, 479–492.

72 Chassot, A. and Richner, W. (2002) *Agron. J.*, **94**, 118–127.

73 Richardson, A.E., George, T.S., Hens, M. *et al.* (2004) Utilization of soil organic phosphorus by higher plants, in *Organic Phosphorus in the Environment* (eds B.L. Turner, E. Frossard and D. Baldwin), CABI Publishing, Wallingford, UK, pp. 165–184.

74 Richardson, A.E., Hadobas, P.A., and Hayes, J.E. (2000) *Plant cell Environ.*, **23**, 397–405.

75 Richardson, A.E., Hadobas, P.A., and Hayes, J.E. (2001) *Plant J.*, **25**, 641–649.

76 Hayes, J.E., Richardson, A.E., and Simpson, R.J. (1999) *Aus. J. Plant Physiol.*, **26**, 801–809.

77 Li, M., Osaki, M., Rao, I.M. *et al.* (1997) *Plant Soil*, **195**, 161–169.

78 Sharma, N.C., Starnes, D., and Sahi, S.V. (2007) *Environ. Pollu.*, **146**, 120–127.

79 Stricker, J.A. (2000) Proceedings of Annual Meeting of the American Society for Surface Mining and Reclamation, Tampa, FL, pp. 644–654.

80 Mertens, J., Luyssaert, S., and Verheyen, K. (2005) *Environ. Pollu.*, **138**, 1–4.

81 Raghothama, K.G. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 665–693.

Part II
Methods to Improve Plant Abiotic Stress Response

Section IIA Introductory Methods

Improving Crop Resistance to Abiotic Stress, First Edition.
Edited by Narendra Tuteja, Sarvajeet Singh Gill, Antonio F. Tiburcio, and Renu Tuteja
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8
Genetic Modification of Crops: Past, Present, and Future*

Nina V. Fedoroff

Agricultural productivity has markedly increased, while the labor intensiveness of agriculture has decreased over the past two centuries of increasing incorporation of science and technology into agricultural practice. Mechanization, chemical fertilizers, plant breeding, and molecular genetic modification (GM) have all promoted rapid increases in agricultural productivity. Recent decades have witnessed a decrease in the rate of agricultural productivity growth, however. The reasons include declining investment in agricultural research, increasing human population pressure, decreasing fresh water availability, increasing temperatures, and societal rejection of GM crops in many countries.

8.1
Introduction

The world has experienced a succession of shocks over the past 2 years: a global food crisis, spiraling energy costs, accelerating climate change, and a financial meltdown. The food crisis sparked riots in countries on every continent [1]. Unfortunately, the food crisis is not a transient phenomenon. The present situation developed over a very long time as a result of relentlessly increasing demand pushing against a shrinking natural resource base, even as investment in agricultural research and development declined decade after decade. The oil price spike combined with widespread droughts in 2007 and 2008 to aggravate the underlying trends and send grain prices upward. While prices moderated in the following year, they have recently renewed their upward trend persists. Indeed, the adequacy of the food supply may be the most critical issue of the twenty-first century [2].

* The views expressed in this chapter should not be construed as representing those of the US government. Fedoroff is on leave from Penn State University, where she is the Willaman Professor of the Life Sciences and Evan Pugh Professor in the Biology Department and the Huck Institutes of the Life Sciences.

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Food security is a long-standing concern. Thomas Malthus' famous essay on population, published in 1798, crystallized the problem of balancing food and human population [3]. Curiously, Malthus penned his essay at about the time when science began to play a major role in boosting agricultural productivity. Late eighteenth-century milestones were Joseph Priestley's discovery that plants emit oxygen [4] and Nicholas-Théodore de Saussure's definition of the chemical composition of plants [5]. Malthus could not have envisioned the extraordinary increases in productivity that the integration of science and technology into agricultural practice would stimulate over the ensuing two centuries.

Both organic and mineral fertilization of plants have been practiced since ancient times. Long before the reasons were understood, people knew that certain chemicals, such as saltpeter and lime, as well as a wide variety of biological materials ranging from fish and oyster shells to manure and bones stimulated plant growth [6]. In the early nineteenth century, Justus von Liebig identified the major chemical requirements for plant growth, laying the foundation for the modern chemical fertilization methods [7]. Although it was known by mid-nineteenth century that biological sources of nitrogen could be replaced by chemical sources, supplying nitrogen in the forms that plants use remained a major limitation until the development of the Haber–Bosch process for fixing atmospheric nitrogen in the early twentieth century [8]. Today, agriculture in the developed world relies primarily on chemical fertilizers.

8.2
Crop Domestication

People practiced what might be thought of as generic "genetic modification" long before chemistry entered agriculture, transforming inedible wild plants into the crop plants that feed people and their animals today. (The term "genetic modification," or "GM," has come most often to refer only to the use of recombinant DNA technology, a confusing state of affairs).

Corn, or maize (*Zea mays*), remains one of humanity's most spectacular feats of genetic modification. Its huge ears, packed with starch and oil, provide one of humanity's most important food and feed crops. Corn bears little resemblance to its closest wild relative, teosinte. Indeed, when teosinte was first discovered in 1896, it was assigned to a different species and named *Euchleana mexicana*. By the 1920s, it was known that teosinte and corn have the same number of chromosomes and readily produce fertile hybrids, yet controversies about their relationship and about the origin of corn continued throughout most of the twentieth century.

The work of Dr. John Doebley and his colleagues, commencing with the genetic analysis of maize–teosinte hybrids, has made substantial progress in identifying the genetic changes that transformed teosinte into modern corn [9]. Doebley's more recent work with evolutionary geneticist Svante Paabo traced the key genetic changes that transformed teosinte into corn to the Balsas River Valley in Mexico and dated them to roughly 6000–10 000 years ago. It has become apparent that the difference between teosinte, a grass with hard, inedible seeds, and modern corn resides in just a handful of

genes that control plant architecture and the identity of reproductive organs. Remarkably, once this handful of mutations were brought together, the suite of genetic modifications stayed together and spread very rapidly, so that the same group of alleles had already penetrated into the American Southwest more than 3000 years ago. Fossilized cobs recovered from caves in Mexico and dated to be more than 6000 year old already possessed the multirowed character of the modern corn ear, as do almost 4000 year-old cobs from the Ocampo Caves in northeastern New Mexico [10].

Perhaps the most important insight that has been gained through the molecular analysis of crop domestication is that people have markedly changed wild plants to make them suitable as crop plants and that this has been done over many thousands of years. They selected phenotypic traits and thereby the underlying genetic changes occurring concomitantly. Each crop has its own interesting history, but one of the most fundamental traits distinguishing wild from domesticated plants is the retention of mature seeds on the plant. Plants have a variety of mechanisms for dispersing their seeds, central to which is the shattering of the seed structure upon maturation. It is much easier for people to harvest seeds that remain attached to the plant, hence the selection of mutations that prevent seed dispersal is thought to be among the earliest steps in crop domestication [11].

Among the many other traits altered during domestication are the size and shape of foliage, tubers, berries, fruits, and grains, as well as their abundance, toxicity, and nutritional value. There are many underlying genetic differences that distinguish a domesticated crop plant from its wild progenitors, but molecular analysis reveals that key changes are often in genes that encode transcription factors – proteins that regulate the expression of many other genes [12]. Differences in nutrient composition among varieties of the same crop are attributable to mutations in genes coding for proteins of certain biosynthetic pathways. For example, mutations in genes for enzymes involved in the conversion of sugar to starch gave rise to sweet corn varieties.

8.3
Modern Crop Improvement

Crop improvement benefited from both the Mendelian and the molecular genetic revolutions of the twentieth century. Austrian monk Gregor Mendel's pioneering observations on inheritance, published in 1865, were made independently by Dutch botanist Hugo de Vries. Only then did Mendel's observations gain the interest of other geneticists [13]. A simple demonstration project to illustrate Mendelian inheritance led to the discovery of hybrid vigor, a phenomenon whose incorporation into crop breeding resulted in a dramatic expansion of the corn ear and, thereby, crop yield. The discovery is attributed to George Harrison Shull, working at the Carnegie Institution of Washington's Station for Experimental Evolution. He was asked by the Station's director to develop a demonstration of Mendel's rules of inheritance. In the course of these experiments, he observed that some kinds of corn made more rows of kernels than others. Curious about the genetic basis of this difference, he inbred the respective varieties and then crossed them to see whether the row number trait would

segregate according to Mendel's simple rules. He found that when he crossed the inbred lines to each other, the F_1 progeny were taller, more robust plants with bigger ears [14]. This phenomenon, called hybrid vigor or heterosis, is the basis of today's extraordinarily productive hybrid corn varieties [15].

However, when they were first introduced in the United States during the 1930s, corn hybrids faced resistance and criticism similar to that of GM crops. The hybrids were complex to produce and agriculture experiment stations weren't interested. Eventually, a company was formed to produce hybrid seed. But farmers accustomed to planting seed from previous year's crop saw no reason to buy it. It was only after farmers realized the yield benefits and the drought resistance of hybrid corn during the 1934–36 dust-bowl years that hybrid corn was rapidly adopted [16].

Techniques for accelerating mutation rates with radiation and chemicals and through tissue culture were developed and widely applied in the genetic improvement of crops during the twentieth century [17]. Such techniques introduce mutations rather indiscriminately and require the growth of a large numbers of seeds, cuttings, or regenerants to detect desirable changes. Nonetheless, all these approaches have proved valuable in crop improvement and by the end of the twentieth century, more than 2300 different crop varieties, ranging from wheat to grapefruit, had been developed using radiation mutagenesis [18].

8.4
Mechanization of Agriculture

A major development the impact of which Malthus could not have envisioned is the mechanization of agriculture. Human and animal labor provided the motive force for agriculture throughout most of its history. Early tractors powered by steam engines were large and unwieldy, but the invention of the internal combustion engine at the turn of the twentieth century led to the development of smaller and more maneuverable machines. The mechanization of plowing, seed planting, cultivation, fertilizer and pesticide distribution, and harvesting accelerated in the United States, Europe, and Asia following World War II [19]. Agricultural mechanization drove major demographic changes virtually everywhere. In the United States, 21% of the workforce was employed in agriculture in 1900 [20]. By 1945, the fraction had declined to 16% and by the end of the century the fraction of the population employed in agriculture had fallen to 1.9%. At the same time, the average size of farms increased and farms increasingly specialized in fewer crops.

8.5
The Green Revolution

Malthus penned his essay when the human population of the world stood at less than a billion. The population tripled over the next century and a half. As the second half of the twentieth century began, there were neo-Malthusian predictions of mass famines

in developing countries that had not yet experienced science- and technology-based advances in agriculture. Perhaps, the best known of the mid-twentieth century catastrophists was Paul Ehrlich, author of *The Population Bomb* [21].

The predicted Asian famines were averted by the dedicated work and extraordinary accomplishments of several scientists and their teams, principally plant breeders Borlaug, Swaminathan, and Khush [22]. The Green Revolution was based on the development of rice and wheat varieties with mutations in genes that controlled their growth rate, resulting in dwarf varieties able to respond to fertilizer application without lodging. Subsequent breeding for increased yield continued to improve the productivity of these crops by as much as 1% per year. Instrumental in these advances were the first two institutes established by the Consultative Group on International Agricultural Research (CGIAR), the International Rice Research Institute (IRRI) [23] in the Philippines, and the International Maize and Wheat Improvement in Mexico (CIMMYT) [24]. Perhaps most remarkably, the Green Revolution of the late twentieth century reduced the fraction of the world's hungry from half to less than a sixth, even as the population doubled from 3 to 6 billion.

8.6
Molecular Genetic Modification of Crops

The molecular genetic revolution that began in the 1960s led to the development of new methods of crop improvement. Researchers in the 1950s and 1960s discovered the existence of bacterial plasmids that could replicate independently of the bacterial chromosome. Other discoveries led to the identification of restriction enzymes and ligases, making it possible to insert and link covalently a piece of genetic material from a completely different organism, then clone (amplify) the plasmid in bacteria. Amplification of such "recombinant" plasmids in turn made it possible to develop the DNA sequencing techniques that underlie today's genomic revolution. Additional techniques were developed for the introduction of genes into plants using either the soil bacterium, *Agrobacterium tumefasciens*, which naturally transfers a segment of DNA into wounded plant cells, or mechanical penetration of plant cells using tiny DNA-coated particles [25]. This combination of techniques has made it possible to transfer genetic material from either the same or a related plant or from a completely unrelated organism into virtually any crop plant.

Several crop modifications achieved using these methods are now in widespread use. Perhaps, the best known of these are crop plants into which was introduced a gene from the soil bacterium, *Bacillus thuringiensis*, long used as a biological pesticide because it produces a protein that is toxic to the larvae of certain kinds of insects, but not to animals or humans [26]. The gene coding for the toxin is commonly called simply "the Bt gene," although there is actually a family of Bt toxin genes that express numerous closely related proteins. These genes have been introduced into a number of different crops, primarily corn and cotton. In the United States and Europe, pest-protected crop varieties are produced almost exclusively by companies such Monsanto, DuPont, and Syngenta. In other parts of the world, including in China and

India, such crop modifications are being performed by both the public and the private research sectors.

Another widely accepted crop modification is the introduction of genes that confer resistance to herbicides, commonly used compounds that inhibit biosynthetic pathways unique to plants [27]. Among the most widely used today are compounds that interfere with the production of amino acids that plants synthesize, but animals do not [28]. Herbicide-tolerant crop plants, which make it possible to control weeds with a herbicide without damaging the crop, have been derived through natural and induced mutations, as well as by introduction of genes from either bacterial sources or modified genes from plant sources. Today, herbicide-tolerant varieties of many crops, most importantly soybeans and canola, are very widely grown.

Papaya varieties resistant to papaya ringspot virus (PRSV) are a public sector GM achievement that saved the $64 million/year Hawaiian papaya industry [29]. Papaya ringspot virus is a devastating insect-borne viral disease that wiped out the papaya industry on Oahu in the 1950s, forcing its relocation to the Puna district of the big island. By the 1970s, the Puna district was producing 95% of Hawaii's papayas. PRSV was first detected in the Puna district in 1992; by 1995 it was widespread and threatened the industry. Dennis Gonsalves and his colleagues at Cornell University began a project in 1985 to introduce a viral gene into papayas based on the observations made in Roger Beachy's laboratory at Washington University that introducing a viral gene could make a plant resistant to the virus from which the gene came [30].

The first transgenic papaya plants expressing a PRSV gene were ready in 1991, small field tests began in 1992 and large-scale field tests began in 1994. Approvals from the Animal Plant Health Inspection Service (APHIS) of the US Department of Agriculture (USDA), as well as the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA), for release of the seeds to farmers took another 3 years, by which time many papaya farmers had gone out of business. Transgenic seeds were released in 1998 and by 2000 the papaya industry had come back to pre-1995 levels. Although it was not known at the time, recent studies have shown that the resistance is attributable to posttranscriptional gene silencing [31]. This remarkable method of crop protection enhances a mechanism present in plants that is responsible for protecting the plant from subsequent infection by the same and closely related viruses, much as the development of immunity protects people and animals from reinfection by pathogens.

8.7 Adoption of GM Crops

Although the use of molecular modification techniques in crop improvement engendered controversy – much of it gratuitous – from the beginning, GM crops have experienced unprecedented adoption rates since their introduction in 1996. By 2008, roughly 10% of cropland was planted with GM crops [32]: transgenic crops were grown on more than 300 million acres in 25 countries by more than 13 million

farmers, 90% of whom were small-holder, resource-poor farmers. The vast majority of transgenic cropland is devoted to just four crops, namely, cotton, maize, soybean, and canola, but the list of commercialized transgenic crops is growing and already includes papaya, tomato, poplar, petunia, sweet pepper, squash, rice, alfalfa, and sugar beet.

Few of the widely anticipated adverse effects have materialized. While some resistance to the Bt toxin has developed, it has not been as rapid as initially feared and second-generation, two-Bt gene strategies to decrease the probability of resistance are already being implemented [33]. Predicted deleterious effects on nontarget organisms, such as monarch butterflies and soil microorganisms, have either not been detected at all or are insignificant. Moreover, while the use of conventional pesticides decreases the abundance of beneficial insects, the planting of Bt crops does not.

The many studies that have been done to assess the safety of foods containing or consisting of GM crops have reached the conclusion that GM foods are as safe or safer than as non-GM foods [34]. This is in part because of the close attention paid during product development to the potential for toxicity and allergenicity of the proteins encoded by genes being added, as well as the inherently greater precision and predictability of GM constructions.

To date, the unexpected effects have been beneficial. For example, many grains and nuts, including corn and peanuts, are commonly contaminated by mycotoxins, toxic compounds made by fungi that follow boring insects into the plants. Two of these, fumonisins and aflatoxin, are extremely toxic and carcinogenic. Bt corn, however, shows as much as a 90% reduction in mycotoxin levels because the fungi that follow the boring insects into the plants cannot get into the Bt plants [35].

There is evidence as well that planting Bt crops reduces insect pressure on other crops growing nearby. Bt cotton has been widely planted in China. Analysis of the population dynamics of the target pest, the cotton bollworm, showed that Bt cotton not only controls the bollworm on transgenic cotton designed to resist this pest but also reduces its presence on other host crops, thereby decreasing the need for insecticide sprays in general [36].

8.8
Future Challenges in Agriculture

The scientific and technological advances in agriculture of the nineteenth and twentieth centuries have been extraordinary. Since Malthus' time, the human population has expanded more than sixfold. In the developed world, agriculture has become far less labor-intensive and has kept pace with population growth worldwide. Today, fewer than 1 in 50 citizens of developed countries grows crops or raises animals for food. This means that most people in developed countries live in cities and find livelihoods that pay higher wages than farming. Those remaining on farms often also work in off-farm jobs, raising average farm income. However, this also means that most citizens of developed countries have little understanding of what it takes to create the foods that stock contemporary supermarkets.

Moreover, after a half century's progress in decreasing the fraction of humanity experiencing hunger from half to less than a sixth, the food crisis and the more recent global financial crisis have begun again to swell the ranks of the hungry [37]. Population experts anticipate an addition of another 2–4 billion people to the planet's population within the next 3–4 decades [38], but the amount of arable land has not changed appreciably in more than half a century, increasing by only about 10% [39] and it is not likely to increase much in the future because we are losing it to urbanization, salinization, and desertification at least as fast as we are adding it.

Another variable that is becoming critical is the availability of fresh water for agriculture. Today, about a third of the global population lives in arid and semiarid areas, which cover roughly 40% of the land area. Climate scientists predict that in coming decades, average temperatures will increase and dry land area will expand [40]. Even now, inhabitants of arid and semiarid regions of all continents are extracting ground water faster than aquifers can recharge and often from fossil aquifers that do not recharge [41].

Thus, the challenges to agriculture in the twenty-first century are profound: to increase agricultural productivity on land largely already under cultivation, at higher temperatures, and using less water. Can it be done? There are biological, political, and cultural impediments.

The major crops that now feed the world – corn, wheat, rice, and soy – require a substantial amount of water. For example, the production of a kilogram of wheat requires between 500 and 2000 L of water, most of which is lost through transpiration [42]. But almost half the grain produced worldwide is fed to animals, and the amount of water required to produce a kilogram of meat is 5–10 times greater than that required to produce a kilogram of grain.

The optimal growth temperature to produce maximal yields of our major crop plants is determined by the temperature optimum for photosynthesis, the process by which plants convert solar energy into chemical energy, and other physiological processes. Yield is also determined by the temperature range that supports optimal development of the harvested storage organs (grain, bean, and kernel) [43]. A recent study reports that yields increase with temperature up to 29 °C for corn, 30 °C for soybeans, and 32 °C for cotton, but then decline precipitously at higher temperatures [44]. This study predicts that yields of these crops in their present growing areas will decline by 30–46% by the end of the twenty-first century under the most moderate climate change scenario and by 63–82% under the most rapid warming scenario.

The expected pressures on water availability and increasing temperatures present critical challenges to agricultural researchers to increase crop water efficiency and heat tolerance. Whether our highly productive food and feed crops can be modified and adapted to be even more productive at the higher temperatures expected or at more northern latitudes is simply not known. It is therefore imperative not only to increase research on the salt, drought, and temperature tolerance of existing crop plants but also to invest in research on plants that are not now used in agriculture, but that are capable of growing at higher temperatures and using brackish or salt water

for irrigation. Indeed, the array of molecular tools and knowledge available today may make it possible to design a wholly new kind of agriculture for a more arid, hotter world.

Even though the molecular tools, physiological knowledge, and genomic information available today are extraordinary, there are also political and cultural barriers to their widespread use in crop improvement. While scientific communities worldwide largely recognize the safety of GM crops, the political systems of Japan and most European and African countries remain opposed to growing GM crops. Many countries lack GM regulatory systems or have regulations that prohibit growing and even, in some countries, importing GM food and feed. Moreover, even where there exist regulatory frameworks that support the testing and introduction of GM crops, the regulatory process is both complex and expensive. Contrary to the claims of anti-GM activists, GM crops and food derived from them are excessively regulated.

These factors have largely eliminated the participation of university and other public sector researchers in molecular crop improvement that involves field trials in most countries around the world. Productivity gains based on earlier scientific advances can still increase food production in some countries, particularly in Africa. But such productivity gains appear to have peaked in most developed countries and recent productivity gains have been made largely through molecular modification. If modern science is to contribute to the agricultural productivity increases required in coming decades as the climate warms and the human population continues to grow, it is imperative to get beyond the cultural and political biases against molecular crop modification, assess the safety record of GM crops, and ease the regulatory barriers to their development and deployment.

References

1 http://www.time.com/time/world/article/0,8599,1717572,00.html.

2 Pinstrup-Andersen, P., Pandya-Lorch, R., and Rosegrant, M.W. (1999) World Food Prospects: Critical Issues for the Early Twenty-First Century, Food Policy Report of the International Food Policy Research Institute.

3 Malthus, T.R. (1798) *An Essay on the Principle of Population, 1798*, 1st edn, anonymous through J. Johnson, London.

4 Priestley, J. (1774) *Experiments and Observations on Different Kinds of Airs*, W. Bowyer and J. Nichols, London.

5 de Saussere, N.-T. (1804) *Recherches Chimiques sur la Végétation*, Nyon, Paris.

6 Hear, F.A. (1938) *Theory and Practice in the Use of Fertilizers*, 2nd edn, Chapman and Hall, London.

7 Liebig, J. (1840) *Organic Chemistry in its Application to Agriculture*, Playfair, London.

8 Russel, D.A. and Williams, G.G. (1977) History of chemical fertilizer development. *Soil Sci. Soc. Am. J.*, **41**, 260–265.

9 http://teosinte.wisc.edu/publications.html.

10 Fedoroff, N.V. (2003) Prehistoric GM corn. *Science*, **302**, 1158–1159.

11 Fedoroff, N.W. and Brown, N.M. (2004) *Mendel in the Kitchen: A Scientist's View of Genetically Modified Foods*, Joseph Henry Press, Washington, DC.

12 Doebley, J.F., Gaut, B.S., and Smith, B.D. (2006) The molecular genetics of crop domestication. *Cell*, **127**, 1309–1321.

13 Carlson, E.A. (1966) *The Gene: A Critical History*, Saunders, Philadelphia.

14 Shull, G.H. (1909) A pure line method in corn breeding. *Am. Breed. Ass.*, Reports **5**, 51–59.

15 Crow, J.F. (1998) 90 years ago: the beginning of hybrid maize. *Genetics*, **148**, 923–928.

16 Crabb, A.R. (1947) *The Hybrid-Corn Makers: Prophets of Plenty*, Rutgers University Press, New Brunswick, NJ.

17 Maluszynski, M., Ahloowalia, B.S., and Sigurbjörnsson, B. (1995) Application of *in vivo* and *in vitro* mutation techniques for crop improvement. *Euphytica*, **85**, 303–315.

18 Committee on Identifying and Assessing Unintended Effects of Genetically Engineered Foods on Human Health (2004) *Safety of Genetically Engineered Foods: Approaches to Assessing Unintended Health Effects*, National Academies Press, Washington.

19 Binswanger, H. (1986) Agricultural mechanization: a comparative historical perspective. *Res. Obs.*, **1**, 27–56.

20 Dimitri, C., Effland, A., and Conklin, N. (2005) The 20th Century Transformation of U.S. Agriculture and Farm Policy, Economic Information Bulletin Number 3, Economic Research Service, USDA.

21 Ehrlich, P. (1968) *The Population Bomb. Ballantine Books*, Random House, New York.

22 Khush, G.S. (2001) Green Revolution: the way forward. *Nat. Rev. Genet.*, **2**, 815–822.

23 http://www.irri.org/.

24 http://www.cimmyt.org/.

25 Birch, R.G. (1997) Plant transformation: problems and strategies for practical application. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **48**, 297–326.

26 http://www.extension.umn.edu/distribution/cropsystems/DC7055.html.

27 www.hort.wisc.edu/cran/pubs_archive/. . ./HowHerbicideWork.pdf.

28 Tan, S., Evans, R., and Singh, B. (2006) Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. *Amino Acids*, **30**, 195–204.

29 Gonsalves, D., Gonsalves, C., Ferreira, S., Pitz, K., Fitch, M., Manshardt, R., and Slightom, J. (2004) Transgenic virus resistant papaya: from hope to reality for controlling papaya ringspot virus in Hawaii. *APSnet*, http://www.apsnet.org/online/feature/ringspot/.

30 Powell, A.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T., and Beachy, R.N. (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science*, **232**, 738–743.

31 Tennant, P., Fermin, G., Fitch, M.M., Manshardt, R.M., Slightom, J.L., and Gonsalves, D. (2001) Papaya ringspot virus resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. *Euro. J. Plant Pathol.*, **107**, 645–653.

32 James, C. (2008) Global Status of Commercialized Biotech/GM crops. ISAAA Brief, 39.

33 Lemaux, P.G. (2009) Genetically engineered plants and foods: a scientist's analysis of the issues (part II). *Annu. Rev. Plant Biol.*, **60**, 511–559.

34 Lemaux, P.G. (2008) Genetically engineered plants and foods: a scientist's analysis of the issues (part I). *Annu. Rev. Plant Biol.*, **59**, 771–812.

35 Munkvold, G.P. (2003) Cultural and genetic approaches to managing mycotoxins in maize. *Annu. Rev. Phytopathol.*, **41**, 99–116.

36 Wu, K.-M., Lu, Y.-H., Feng, H.-Q., Jiang, Y.-Y., and Zhao, J.-Z. (2008) Suppression of cotton bollworm in multiple crops in China in areas with Bt toxin-containing cotton. *Science*, **321**, 1676–1678.

37 FAO (2008) Hunger on the Rise: Soaring Prices add 75 million People to Global Hunger Rolls. Briefing paper, FAO, Rome.

38 Cohen, J.E. (2003) Human population: the next half century. *Science*, **302**, 1172–1175.

39 The Land Commodities Global Agriculture and Farmland Investment Report (2009) www.landcommodities.com.

40 Climate Change 2007: Impacts, Adaptation and Vulnerability, IPCC Fourt Assessment Report http://www.ipcc.ch/publications_and_data/

publications_ipcc_fourth_assessment_report_wg2_report_impacts_adaptation_and_vulnerability.htm.

41 Giordano, M. (2007) *The Agricultural Groundwater Revolution: Comprehensive Assessment of Water Management in Agriculture*, International Water Management Institute.

42 Water Policy Briefing 25, International Water Management Institute; http://www.unwater.org/downloads/WPB25.pdf.

43 Qaderi, M.M. and Reid, D.M. (2009) Crop responses to elevated carbon dioxide and temperature, in *Climate Change and Crops* (ed. S.N. Singh), Springer, pp. 1–19.

44 Schlenker, W. and Roberts, M.J. (2009) *Proc. Natl. Acad. Sci. USA*, **106**, 15594.

9
Translational Biology Approaches to Improve Abiotic Stress Tolerance in Crops

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In the last decades, several genes that regulate abiotic stress response were identified in the model plant *Arabidopsis*. The completion of the *Arabidopsis* genome sequence in 2000 and the development of molecular high-throughput "omics" platforms have speeded up the simultaneous functional characterization of genes that regulate the abiotic stress tolerance in plants. The fundamental molecular mechanisms that underlie the plant abiotic responses are quite conserved among plant species and therefore the knowledge gained in the model plant *Arabidopsis* can be exploited to improve stress tolerance in crop species. Many examples demonstrate that ectopic expression of key genes involved in the stress response can induce stress tolerance in different crops, demonstrating the potential of the translational approaches. Nevertheless, generation of crops that have improved tolerance under field conditions is still a challenge. However, systems biology studies in model plants and "omics" approaches in crop species can certainly contribute to the understanding of the interplay between response pathways to different abiotic stresses, a condition that is commonly found in open fields.

9.1
Introduction

Plants represent our main food supply both directly or indirectly via the production of feed for bred animals. The agricultural production greatly increased during the Green Revolution thanks to the introduction of new cultivars and the use of fertilizers and pesticides. The gene revolution, which started with the use of genomic and biotechnology approaches for plant genetic improvement, was expected to give a big speedup in the selection of new varieties. However, the development of an ideal crop for different environments is still a challenge. Plant growth and production are greatly influenced by the environment and particularly by climate stresses. The global climate changes that we are experiencing in recent years is worsening the losses of

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plant production. The higher frequency of long drought periods alternated with flooding, which are becoming common even in temperate regions, increases the unpredictability of crop yield.

Plants can afford mild abiotic stresses putting in place molecular mechanisms that ensure survival and reproduction. Since sessile, plants have evolved the ability to reprogram the expression of their genome in response to environmental changes. However, survival under abiotic stresses is costly in terms of energy and metabolism. The adverse effect of abiotic stresses is reflected on plant health, *habitus*, and production. It is estimated that abiotic stresses account for 70% reduction in the yield for main crops [1]. Several pathways that regulate the changes in metabolism, upon environmental stresses, were studied in detail in model plants. However, there is need to "translate" the fundamental studies, conducted on model plants, into applied research for crop improvement [2].

The concept of "translational biology" is borrowed from medicine where there is an urgent need to translate into practical protocols discoveries coming from the fundamental science. Nevertheless, this approach might have a useful application in agriculture as well, and can be translated as the use of knowledge developed in model plants (*Arabidopsis thaliana*, *Indica rice*, *Medicago truncatula*, *Populus* spp., etc.) for crop genetic improvement. The recent progresses made in model plants in the field of genomics, transcriptomics, and proteomics can give great opportunities for the genetic improvement of crops.

On the other hand, the swift adoption of next-generation sequencing (NGS) technologies, which are producing reference genome data directly on crops, will allow the development of comparative genomic approaches with model plants in order to find new genes and new markers useful for breeding traits of interest. A big discrepancy between the vast quantity of data already available at genomic DNA sequence level and the information regarding the function of genes and meaningful phenotypes still exists. This limitation hampers the possibility of fully exploiting the potential of genomics tools for breeding, such as genome-wide association and genomic breeding.

An exciting area of development, which very likely will allow to tackle this problem, is represented by high-throughput plant phenotyping, the so-called plant phenomics, which is aiming at the development of platforms that support fast and efficient phenotype screening in mutants, populations, and their relations with environmental factors.

9.2 *Arabidopsis* as a Model System

During the past 10 years, *Arabidopsis* has underpinned the genomic revolution in plant science. A number of powerful genomic tools have been developed: a high-quality genome sequence that now includes detailed information on whole-genome patterns of DNA methylation, histone modifications, and small RNAs; T-DNA

insertions in around 90% of genes; whole-genome expression profiles at different development stages in a wide range of environments (www.arabidopsis.org; January 15, 2011). Largely through the use of these genomic tools, *Arabidopsis* research has in the past decade made substantial progress in identification of key genes that underlie the major processes of plant development and responses to the environment. As an example, here we describe the recent advances made in understanding the transcriptional regulatory networks and upstream regulators in response to cold and drought.

9.2.1 Cold Stress

In *Arabidopsis*, *CBF/DREB* (C-REPEAT-BINDING FACTOR/DEHYDRATION-RESPONSIVE ELEMENT BINDING) genes are rapidly and transiently induced following exposure to low temperature, and the CBF/DREB factors in turn induce the expression of around 100 other genes, collectively termed the CBF regulon, which is central to cold acclimation (see Refs [3, 4] and references therein). Recent work identified two cold-sensing pathways that control expression of *CBF/DREB* genes. ICE1 (INDUCER OF CBF EXPRESSION 1), a basic helix-loop-helix transcription factor, binds to multiple Myc DNA regulatory elements present in the *CBF3/DREB1a* promoter and stimulates *CBF3/DREB1a* transcription (see Refs [3, 4] and references therein). The *ICE1* gene is constitutively expressed and regulated at the posttranslational level through low-temperature-induced sumoylation of the ICE1 protein mediated by SIZ1, a SUMO E3 ligase [5]. Interestingly, this activation process is countered by HOS1 (High Expression of Osmotically Responsive Genes 1), a RING finger E3 ligase that mediates ubiquitination and degradation of ICE1 (see Ref. [4] and references therein). The other cold-sensing pathway involves calcium. A number of studies have established that exposure of plants to low temperature induces calcium spikes that in turn influence gene expression (see Ref. [4] and references therein). Recently, Doherty *et al.* [6] showed that calmodulin binding transcription activator (CAMTA) factors bind to a regulatory element in the *CBF2/DREB1c* gene promoter. Single *catma* mutants showed no obvious growth phenotypes. However, the *camta3* mutation resulted in a significant reduction in cold induction of *CBF2/DREB1c*, and *camta1 camta3* double-mutant plants were impaired in freezing tolerance, indicating an important role the CAMTA transcription factors play in cold acclimation [6].

Cold induction of *CBF/DREB* genes is gated by the circadian clock: plants exposed to cold at ZT4 (4 h after dawn) have greater induction of *CBFs/DREBs* compared to plants given the same treatment at ZT16 [7]. Intriguingly, the CBF pathway is also controlled by light quality [8]. A low red to far red (R/FR) ratio light signal at the beginning of the day induced the *CBF/DREB* genes and their downstream *COR* (cold-regulated) genes at 16 °C but not at 22 °C. Remarkably, this low R/FR-induced increase in *CBF/DREB* expression was sufficient to confer freezing tolerance at 16 °C. The reduced ambient temperature together with a low R/FR light environment

at the beginning of the day might mimic autumn, and it is possible that the activation of the CBF/DREB regulon represents an adaptive response in *Arabidopsis* for minimizing winter freezing injury [3, 8].

Natural variation studies further highlighted the regulatory role of *CBF/DREB* genes in cold acclimation. Natural accessions of *A. thaliana* are distributed over a broad geographic range where selection pressures for tolerance to low temperature are diverse [9]. Relative to accessions from northern regions, accessions of *Arabidopis thaliana* from the southern part of their geographic range exhibit higher levels of nucleotide polymorphism in both regulatory and coding regions. Relaxed selection on the *CBF/DREB* genes in southern accessions compromised the ability of these genes to act as efficient transcriptional activators during cold acclimation [10].

Transgenic overexpression of individual *CBF/DREB* genes induces the cold acclimation pathway and results in enhanced freezing tolerance in the absence of a cold acclimation treatment [11]. However, *Arabidopsis* plants constitutively overexpressing *CBF1/DREB1b*, *CBF2/DREB1c*, or *CBF3/DREB1a* grow slowly, have a dwarf stature, and have delayed flowering [11, 12]. Recently, Achard *et al.* [13] discovered that constitutive overexpression of *CBF1/DREB1b* results in the accumulation of DELLA proteins (DELLAs), a family of nuclear growth-repressing proteins that are key components of the gibberellin (GA) signaling pathway (see Ref. [14] and references therein). According to the current model, DELLAs restrain plant growth, whereas GA promotes growth by overcoming DELLA-mediated growth restraint (see Ref. [14] and references therein). The binding of bioactive GAs to the nuclear receptor GID1 (GA-INSENSITIVE DWARF1) promotes an interaction between GID1 and the DELLA domain of DELLAs. Subsequently, this interaction enhances the affinity between DELLAs and a specific SCF E3 ubiquitin–ligase complex involving the F-box protein SLY1 (SLEEPY1), thus targeting DELLAs for destruction by 26*S* proteasome (see Ref. [14] and references therein). Achard *et al.* [13] found that overexpression of *CBF1/DREB1b* enhances expression of GA-inactivating *GA 2-oxidase* genes. Accordingly, transgenic plants that constitutively express *CBF1/DREB1b* accumulate less bioactive GA and as a consequence exhibit dwarfism and late flowering. Both phenotypes are suppressed when *CBF1/DREB1b* is expressed in a line lacking two DELLA proteins, GAI (GA-INSENSITIVE) and RGA (REPRESSOR OF GA1).

The CBF/DREB cold response pathway is highly conserved in plants. Components of this pathway are also present in species that suffer chilling injury at low temperatures and are unable to tolerate freezing, such as tomato, rice, and maize [15, 16]. Targeted manipulation of these regulatory genes may be able to improve freezing tolerance in winter crops [17].

9.2.2
Drought Stress

Plant responses to drought are complex and different pathways have been identified in *Arabidopsis* (see Ref. [18] and references therein). Among them, the

abscisic acid (ABA) signaling pathway plays a crucial role in plant survival under drought stress. Drought triggers the production of ABA that in turn causes rapid stomatal closure to limit water loss through transpiration and induces genome reprogramming to survive this adverse environment (see Ref. [19, 20] and references therein).

Several ABA binding proteins have been identified and implicated in ABA signaling (see Ref. [19] and references therein). However, the recent identification of the PYR/PYL/RCAR PYRABACTIN (4-bromo-N-[pyridin-2-yl methyl]naphthalene-1-sulfonamide) RESISTANCE (PYR)/PYRABACTIN RESISTANCE 1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) family of ABA receptors and their mechanism represents a major breakthrough in the field and may open up the possibility of new approaches to improve drought tolerance in crops [21–24]. Three research groups independently identified different members of this new receptor family as ABA binding proteins that interact with clade A protein phosphatase 2Cs (PP2Cs), which function as negative regulators of ABA signaling [22–24]. Group A PP2Cs consists of nine proteins including ABA insensitive 1 (ABI1), ABI2, and homologue of ABI1 (HAB1), very well known for their role in ABA response (see Ref. [19] and references therein). The use of ABI2 and HAB1 as baits in yeast two-hybrid screens led to the identification of PYL9/RCAR1, PYL8, and PYL5 as PP2C binding proteins [22, 23]. Independently, Nishimura *et al*. [24] identified several PYR/PYL/RCAR proteins as interactors of ABI1 *in vivo*. Taking a different approach, Park *et al*. [21] identified pyrabactin, a new synthetic selective ABA agonist, and determined by genetic analysis that PYR1 is necessary for pyrabactin action *in vivo*. Subsequent *in vitro* studies demonstrated that both pyrabactin and ABA cause PYR1 to bind and inhibit ABI1, ABI2, and HAB1 [21].

The *Arabidopsis* genome encodes 14 highly conserved PYR/PYL/RCAR proteins [25] belonging to the START/Bet v I superfamily that contain a central hydrophobic ligand binding pocket [26, 27]. The structures of PYR1, PYL1, and PYL2 in apo, ABA-bound, and ABI1 or HAB1 complexed forms were recently reported and revealed how ABA binding to PYR/PYL/RCAR receptor proteins leads to PP2C inhibition [28–32].

Although the single *pyr/pyl/rcar* mutants characterized so far do not display ABA phenotypes, a quadruple *pyr1/pyl1/pyl2/pyl4* mutant shows defects in several ABA responses demonstrating a major role of the PYR/PYL/RCAR proteins in ABA signaling [21, 24]. Moreover, transgenic plants overexpressing *RCAR1/PYL9* are hypersensitive to ABA-promoted guard cell closure [22], and overexpression of *PYL5* enhances drought tolerance of transgenic *Arabidopsis* plants highlighting the biotechnological potential of this gene [23].

In the past years, several binding partners of PP2Cs have been identified (see Ref. [19] and references therein). Among them are protein kinases implicated as positive regulators of ABA signaling such as OST1 (Open Stomata 1)/SnRK2.6 (SNF1-related kinase 2.6) [33, 34] and SnRK2.2 [21]. Triple mutants lacking OST1/SnRK2.6 and its two close relatives SnRK2.2 and SnRK2.3 are almost completely unresponsive to ABA, indicating that these kinases are central to ABA signaling [35–37]. Accumulated evidence indicates that group A PP2Cs directly inhibit SnRK2

kinase activity by dephosphorylating them [38–40]. Therefore, the PYR/PYL/RCAR-mediated inhibition of PP2C activity results in SnRK2 kinase activation, which in turn leads to phosphorylation of downstream targets including AREB/ABFs transcription factors that bind to ABA-responsive promoter elements (ABREs) (see Ref. [21, 41] and references therein). Remarkably, the core PYR/PYL/RCAR–PP2C–SnRK2 pathway, originally proposed by Park *et al*. [21], is sufficient for mediating an ABA response. ABA-triggered phosphorylation of the ABRE binding transcription factor AREB1/ABF2 was reconstituted *in vitro* by combining recombinant PYR1, ABI1, and OST1/SnRK2.6 proteins [42].

The PYR/PYL/RCAR receptor family is well conserved in crop species; thus, it is likely that the modulation of these proteins and their interacting partners will enable new strategies to increase crop tolerance both to drought and to other stresses [43, 44]. Moreover, the discovery of a selective ABA agonist, pyrabactin, and the understanding of its structural mechanism of action and selectivity point to a potential chemical strategy for improving crop yield under drought stress [21, 45, 46].

9.3 Abiotic Stress Tolerance in Crops

During the process of selection of crops that was made in the last century, most attention was devoted to productivity, while abiotic stress tolerance traits were not considered intensively. Nowadays, we are facing a period of progressive drought, usually accompanied with increasing salinization, all around the world so that regions that were considered temperate or with good water resources are becoming dry regions. Furthermore, the global population is projected to increase in the next 30 year from 6.7 billion to 9 billion (http://timeforchange.org/growing-world-population; February 28, 2011) [47], so the need for increasing crop productivity is becoming more and more urgent. Drought stress causes severe losses in plant biomass and seed production. It is estimated that more than 50% of the potential production is lost to drought periods (see Ref. [48] and references therein).

Plants can face drought stresses accumulating in their tissue water either by absorbing more efficiently water from the soil by expanding the length of roots or by decreasing the loss of water via the reduction of the stomatal aperture. In contrast, drought resistance plants are able to limit their metabolism to survive in extreme drought and restore their biological functions once the environmental conditions such as water availability are restored. An example of drought-resistant plant is the *Craterostigma plantagineum*, also called resurrection plant, which has been intensively investigated to understand the molecular mechanisms that regulate the stress response [49].

In recent years, significant progress has been made to improve the abiotic stress tolerance in crops using conventional breeding, marker-assisted breeding, and genetic engineering (reviewed in Ref. [50]). The unraveling of *Arabidopsis* genome has allowed identification of key genes in this model plant that were

demonstrated to control many transcriptional regulatory networks as described in the previous paragraph.

In the following section, the progress made in recent years to obtain stress-tolerant crops (mainly to drought, salt, and cold) is summarized.

Several crops have been engineered to cope with water shortage. Attention was focused on important crops such as tomato, tobacco, wheat, rice, barley, sugarcane, corn, and rape using genes codifying for the production of organic solutes, plant growth regulators, antioxidants, late embryogenesis proteins (LEAs), heat shock factors (HSFs), and heat shock proteins (HSPs). Furthermore, the use of transcription factors (TFs) that regulate the expression of several genes has been investigated (see for recent reviews Refs [18, 50–52]).

The one-gene approach was broadly used in the past 20 years to obtain plants with better performances under stress. The identification of genes coding for water-deficit-induced genes started in the early 1990s [53], and in the following years many of them were transferred to crop's genome. Attention was focused on osmoprotectants (proline, glycine betaine (GB), and trehalose) and several crops were transformed with genes encoding compatible osmolytes [54–58]. Compatible solutes are small molecules that are highly soluble in water and have no effect on macromolecules even at high concentration [59]. One of the main actions of osmolytes is to maintain the turgor pressure in the presence of dehydration, which is the first effect of drought, salt, or heat stress.

9.3.1 Proline

Among osmolytes, proline is the most investigated and several data on its role during stress, signaling, and development have been unraveled [60–62]. Genes codifying proline were isolated from plants (*Vigna aconitifolia*) or bacteria (*Escherichia coli*) and transferred into crops. The first evidence that proline overexpression renders tolerance to osmotic stress was demonstrated by Kishor *et al.* [54]. In this work, tobacco plants constitutively overexpressing mothbean (*V. aconitifolia*) P5CS (Δ^1-*pyrroline-5-carboxylate synthetase*), which is the rate-limiting enzyme in proline biosynthesis, showed greater root biomass and higher number of capsules under salt stress. Furthermore, the overexpression of a mutated form of P5CS (P5CSF129A), in which the feedback regulation by proline was impaired, resulted in an increased production of proline under stress. Transgenic seedlings were able to grow on medium containing 200 mM NaCl, and the level of proline in seedlings expressing P5CSF129A was twofold compared to P5CS seedlings [63]. However, it was also suggested that the role of proline during stress is not limited to osmolytes, but this amino acid acts as a free radical scavenger. The *P5CS* gene from mothbean was also inserted into the rice genome under transcriptional control of the ABA-inducible promoter complex (*AIPC*) by Zhu *et al.* leading to rice plants that showed an increased root biomass under drought stress and less severe wilting compared to the control [55]. A similar result was obtained with the *P5CSF129A* gene that lacked the

feedback control as discussed earlier [64]. In this case, transgenic plants accumulated more proline (four- to fivefold compared to the wild type), were more resistant to salt stress, and showed lower lipid peroxidation, confirming the role of proline as osmoprotectant and as free radical scavenger. The accumulation of proline can be achieved by inhibiting the proline degradation via the antisense regulation of *PDH* (proline oxidase). However, contrasting results were obtained using this approach. While Nanjo *et al.* [65] found an increased tolerance to salt and cold in *Arabidopsis*, other studies highlighted an abnormal plant development and almost no influence on the plant ability to cope with stress [66]. By comparing these results, it can be argued that the rate of proline biosynthesis is more critical than the proline content for stress tolerance.

9.3.2 Glycine Betaine

GB is a quaternary compound widely distributed in various organisms that accumulates in chloroplasts and plastids of many plants. High levels of GB are found in halotolerant plants under stress conditions [67]. However, GB has multiple roles in the cell during abiotic stress: it acts as osmoprotectant maintaining the water balance between the cell and the environment, helps in membrane protection, and stabilizes the quaternary structure of enzymes and proteins at nonphysiological temperatures and salt concentrations [68]. The production of GB in many plants occurs via a two-step oxidation of choline via betainealdehyde involving the choline monoxigenase (CMO) and betainealdehyde dehydrogenase (BADH) [69], while in some extremophile microorganisms an alternative pathway of GB synthesis, via a three-step methylation of glycine, was identified [70]. Although most plants accumulate GB, some of them, including *Arabidopsis*, tobacco, and rice, are considered nonaccumulating. Both naturally accumulating GB and nonaccumulating plants were genetically transformed with GB codifying genes isolated from various organisms such as *E. coli* (*BADH*, *CDH*, [71] *BetA*, *BetB* [72]), *Arthrobacter globiformis* [73, 74], and plants (*CMO* [75] and *PEAMT* [76] from *Spinacia oleracea*). Transgenic crops accumulating GB in their cells showed an increased tolerance to several injuries (drought, salt, and cold). However, the level of accumulation of GB in transgenic plants was not very high and the maximum amount of 5.7 $\mu mol\ g^{-1}$ FW was obtained in *betA* transgenic maize [77, 78]. These plants were found resistant to drought and cold compared to their wild-type counterpart and showed a greater grain production under drought conditions. Accumulated evidence demonstrates that targeting GB in distinct cell compartments leads to different effectiveness in protection against environmental injuries. For instance, in the study by Park *et al.* [79], the *A. globiformis CodA* gene was targeted at cytosol or at chloroplasts and at both compartments simultaneously; the highest levels of GB were obtained in plants that accumulated GB in the cytosol, but the protection against salt and cold stress was much effective in transgenic lines in which the GB gene was targeted at chloroplasts, in spite of the low amount of GB obtained in this subcellular compartment. The authors argued that the presence of GB in chloroplasts reduces the detrimental effect of ROS and enhances tolerance of PSII to photoinhibition.

9.3.3 Transcription Factors

The approach involving the use of TFs is likely to be one of the most promising to obtain an effective abiotic stress resistance in crops. TFs are involved in several basic plant metabolisms and regulate entire gene networks that are involved in plant response to biotic and abiotic stresses. In the history of plant domestication, TFs have played a great role allowing selection of plants more suitable for agriculture purposes. Take, for instance, the case of the selection of maize from teosinte: the selection of a mutant altered in the regulation of the *Tb1* (*teosinte branched 1*) allele (that regulates the lateral branching determining the outgrowth of branches when it is expressed) resulted in a dramatic change in the architecture of the progeny starting the domestication of the modern corn [52, 80].

Several TFs belonging to different large protein families (e.g., HD-Zip, AP2/EREBP, MYB, and NAC) have been identified and overexpressed in *Arabidopsis* [81–86]. Jung *et al.* found the TF gene *AtMyb44* was either overexpressed or downregulated in transgenic *Arabidopsis* and the tolerance of these plants to drought and salt was verified. The authors observed a plant survival rate of 82% for AtMyb44 overexpressing plants, 17% for wild type, and 8% for *atmyb44* downregulated plants, demonstrating a major role of this TF in preventing water loss under drought stress. Furthermore, roughly the same survival rate was recorded in experiments in which transgenic plants were watered with increasing concentration of NaCl (up to 300 mM).

However, TFs isolated in *Arabidopsis* have been transferred to crops to verify their action in heterologous systems. It is quite clear that in many cases the *Arabidopsis* TFs are able to modulate the expression of crop genes involved in the abiotic stress responses [87, 88]. Most attempts have been performed using *Arabidopsis* genes belonging to the *CBF* family since *CBF1-like* ESTs were previously identified in several crops including tomato [15]. Hsieh *et al.* found out that the ectopic expression of *Arabidopsis CBF1* cDNA was able to confer drought, chilling, and oxidative stress tolerance in transgenic tomato plants [87, 89]. In the drought trial, transgenic tomatoes were able to survive after 4 weeks of water deprivation, while the wild-type counterpart was not. In the cold experiment, a very high percentage (ranging from 75% to 83.3%) of transgenic plants was able to recover after 7 days at 0 °C in contrast to the wild-type plants that eventually died. However, the cold-tolerance plants exhibited a dwarf phenotype and reduced seed number probably caused by the constitutive expression of *CBF1*. The alteration in the growth of transgenic plants was overcome by exogenous GA_3 treatment, suggesting that CBF1 may influence genes coding for hormones involved in plant growth. Importantly, the use of *CBF1* demonstrates that the ectopic expression of key regulatory genes can confer tolerance to multiple stresses in relevant crops probably due to the crosstalk of different stress signaling pathways. Recently, it has been suggested that the chilling tolerance observed in CBF1 overexpressing plants could be imputed to the protection of the photosynthetic activity during cold stress [90]. In fact, the *Arabidopsis CBF1* gene was transferred in a cold-sensitive tobacco

cultivar and the photochemical function of PSII and PSI under low irradiance was evaluated. It was found out that all parameters relative to photochemical efficiency (Fv/Fm, NPQ, and ETR) were more favorable in transgenic lines compared to control, thus showing a protective mechanism of CBF on the activity of chloroplasts.

In the last decade, the availability of the genome sequence for several crops allowed identification of *Arabidopsis* TF orthologues in important plants such as rice, tomato, wheat, and maize. Overexpression of rice *CBF/DREB1A* orthologues (*OsDREB1A* and *OsDREB1B*) in *Arabidopsis* gave rise to transgenic plants with better ability to cope with drought, salt, and freezing stresses [91]; in addition, the overexpression of the same genes in rice led to the obtainment of transgenic plants with enhanced tolerance to drought, salt, and cold stress [92].

Lately, AREB-like transcription factors (abscisic acid-responsive element binding factor) were used by two different groups to obtain drought-resistant tomato [93, 94]. *SlAREB1* and *SlAREB2* were isolated from tomato and constitutively overexpressed in the same species. Plants showed a high degree of drought and salt tolerance and were phenotypically equivalent to wild type. Microarray analysis revealed that several abiotic stress-regulated genes were upregulated and, more importantly, genes related to the biotic stress response were upregulated as well, suggesting a crosstalk between biotic and abiotic stress response in plants.

However, in several studies it was observed that the constitutive overexpression of transcription factors caused phenotypic changes in the regenerated plants (e.g., stunted plants, delayed flowering, and reduced seed set) [92, 95, 96], though there are some exceptions in which transgenic plants showed a normal phenotype [88, 93, 97]. To reduce the influence of the exogenous gene on plant phenotype, many researchers expressed the gene of interest under transcriptional control of stress-inducible or tissue-specific promoters. An ideal promoter should have a tight regulation and it should be rapidly and transiently activated upon stress. These characteristics are typical of the *HSP* genes in which the transcription is regulated by heat shock elements present in the upstream region of the *HSP* genes [98]. However, only few examples are available on the use of *HSP* promoters for the transcriptional regulation of stress-related genes [99, 100]. The use of stress-related genes under transcriptional control of inducible promoters may minimize the adverse effect of the exogenous gene at phenotypic level. Kasuga *et al*. compared the growth of transgenic plants containing the CaMV35S :: DREB1A and *29A* :: DREB1A [101] and observed that the use of the *rd29* promoter allowed regeneration of tobacco plants that were almost not affected in their growth and showed a comparable level of drought and cold stress tolerance compared to the CaMV35S :: DREB1 transgenic plants. Lee *et al*. transformed tomato cotyledons with *CBF1* driven by the stress-inducible promoter *ABRC1* isolated from barley and obtained transgenic plants with enhanced tolerance to chilling, drought, and salt stress. Transgenic plants did not show any growth retardation and, notably, the fruit production in transgenic tomatoes was comparable with wild-type plants under nonstress condition and better under the above-mentioned stress conditions [102].

9.4 From Arabidopsis to Crop

The convergence of research effort in *Arabidopsis* and in other model systems is generating a knowledge base and variety of valuable tools that are amenable to crop research. The main abiotic stresses that adversely affect plant growth and productivity are being extensively studied and include drought, salinity, heat, cold, chilling, freezing, nutrient, high light intensity, ozone, and anaerobic stresses (reviewed in Ref. [18]).

Based on the examples discussed in this chapter, it seems reasonable to anticipate that TF-based technology will be a significant component of near-future agricultural biotechnology products, conferring enhanced intrinsic yield and yield stability under stress conditions. Of course, a detailed understanding of the response of plants to abiotic stress is a prerequisite to the discovery and use of key regulatory molecules to activate a balanced acclimation response that will enhance the tolerance of plants to different stresses. Various strategies can be used to enhance the tolerance of plants to abiotic stress by genetic engineering. Strategies for the use of selected genes in crops include gain- and loss-of-function approaches that target single genes at various levels. However, as discussed in the previous paragraphs, constitutive ectopic expression of these components often causes reduced plant growth, presumably due to an adverse effect of accumulated molecules on cellular functions or energy consumption. The improved tolerance to abiotic stresses could be facilitated during normal stress episodes in the field via the use of tissue-specific or abiotic stress response promoters [103]. Another promising strategy is the use of chemical-specific inducible promoters driving the expression of regulatory factors relevant for stress adaptation. For instance, the improved tolerance to certain abiotic stresses could be triggered prior to the stress event using different chemicals, a strategy similar to the priming used to alleviate biotic stresses. The nonprotein amino acid, β-aminobutyric acid (BABA), a potent inducer in plants against microbial pathogens, was found to increase drought, salt stress tolerance [104], and thermotolerance in *Arabidopsis*. In addition, thiourea treatment coordinately regulates different signaling and effector mechanisms in *Brassica juncea* at an early stage to alleviate stress even under a high degree of salinity [105]. This also indicates the potential of thiourea to be used to impart salinity tolerance under field conditions. Therefore, the same chemical could be used both as an effective bioregulator to impart stress tolerance under field conditions and as an inducer of chemical-specific inducible promoters driving the expression of selected TFs.

Another strategy for optimizing the phenotype is by protein modification. Some transcription factors may be converted to constitutive active forms by deleting the inhibitory domains or changing phosphorylation-accepting amino acid residues to phosphorylation-mimicking amino acid residues (e.g., serine to asparatic acid). In addition, transcription factors may be converted to dominant negative forms either by fusing a repressor domain to the protein (e.g., the EAR repressor domain) [106] or by removing the DNA binding domain when acting in homo- or heterodimeric

complexes [107]. A different approach to introduce specific amino acid changes by point mutation in the coding sequence is the TILLING technology (see Ref. [108] and references therein).

In some cases, stress resistance may also be conferred by gene downregulation. This may be achieved by RNA interference [109], cosuppression [110], or loss-of-function mutants [111–113]. Artificial microRNAs (amiRNAs) may be an alternative strategy to knockdown the expression of specific genes. The major role of short single-stranded RNA molecules (miRNAs) in stress responses has been elucidated only recently and is reviewed in Ref. [114].

To better understand plant stress responses, transcript profiling experiments have been successfully carried out for many abiotic and biotic stresses [115–120]. One common theme emerging from microarray analysis is that the response initially is composed of a core set of multistress responsive genes and becomes gradually more stress specific as time progresses. Significantly, key molecular components of the multistress responsive genes have been shown to be evolutionarily conserved in all organisms [121]. Furthermore, the *Arabidopsis* multistress responsive genes contain in their promoters a DNA motif that is sufficient to confer a rapid response to both biotic and abiotic stresses *in vivo*. This *cis*-regulatory element may be useful for the construction of artificial stress-inducible promoters to drive the expression of specific TFs [120].

Plant acclimation to a particular abiotic stress condition requires a response adapted to the precise environmental condition that the plant encounters [122]. Therefore, it is not surprising that biochemical, physiological, and molecular events triggered by a specific environmental stress are different from those caused by a different set of abiotic factors [123–126]. However, combined abiotic stresses have been reported to cause unpredicted physiological changes in plant cells [122, 127]. Drought and heat stress represent an excellent example of two distinct environmental stress conditions that occur together in the field [122]. When these stresses are applied together, the physiological and metabolic response of plants is unique and cannot be directly extrapolated from the response of plants to drought or heat stress applied individually [125–127]. Transcriptome profiling of *Arabidopsis* plants indicated that the acclimation response involved changes in gene expression, with more than 400 transcripts specifically expressed in plants during a combination of drought and heat stress [126]. Distinct responses were also observed in plants exposed to a combination of heat and high light intensity [128] and heat and salinity [129]. In addition, abiotic stress and ABA responses interact with the defense response against pathogens in a highly intricate manner (reviewed in Ref. [130]).

At this point, it should be evident that physiological knowledge of the processes of abiotic stress tolerance is still developing, and significantly more effort needs to be invested to both complement and guide breeding and genetic manipulation programs. For instance, the root:shoot ratio has been shown to be an important determinant in the ability of plants to respond to environmental stress, and in the field the root systems is a fundamental component in the plant strategy for stress avoidance [131]. The genetic complexity and the difficulty in scoring and selecting traits affecting the development of this organ that grows below the surface of the soil

have made it very complex to breed for improved root systems. Very recently, it has been shown that the root-specific expression of genes encoding cytokin-oxidase (CKX) enzymes in *Arabidopsis* and tobacco allows the production of transgenic plants with an enlarged root system without negative side effects on aerial organs. Interestingly, these CKX transgenic plants showed a higher survival rate after severe drought treatment [132]. Therefore, it would be very interesting to test the performance of CKX transgenic plants experiencing drought stress in the field.

So far, in many studies describing stress tolerance of mutants or genetically manipulated plants, the levels of plant tolerance against a stress were examined only over short periods. Consequently, crops harboring transgenes or mutated genes designed to improve the tolerance to a specific stress might encounter unexpected problems when grown in the field. The plant should be always tested under experimental settings that mimic the various combinations of restrictive conditions that occur in the field [18]. However, these are time-consuming experiments, especially for testing new hypothesis. The multiplicity of stressing environments, difficult to handle, can be better addressed by systems biology approaches [133] owing to the development of models [134–138]. Of course, the final goal would be to understand the molecular mode of action of the whole stress response system of plants.

High-throughput experiments in model plant systems can already provide us with essential, albeit partial, information. Integration of transcript profiling with other multiple "omics" (e.g., metabolic and proteomic profiling) and phenotypic data is required to reconstruct complex networks and to identify key regulatory steps that characterize the response of the plant under changing environmental conditions. However, it is important to point out that to date most of the key components are still unidentified. In *Arabidopsis*, the molecular function, biological process, or cellular compartment of approximately 30% of the genes is known and a further 40% have mere experimental annotations in the *Arabidopsis* database of genetic and molecular biology data (TAIR, www.arabidopsis.org; January 15, 2011). The situation is worse with respect to metabolites, even in the *Arabidopsis* model. Of the estimated 10 000 metabolites in *Arabidopsis* [139], only approximately 1000 have been structurally resolved [140–142]. In addition, it has been shown that abiotic stress induces the accumulation of novel antisense overlapping transcripts [143] and transcripts from transposons or pseudogenes, which are a source of siRNAs [144, 145], implying a role for siRNAs in abiotic stress responses in plants. Recent studies have also shown that abiotic stresses cause long-term regulation of gene expression, mostly conferred by epigenetic gene regulatory mechanisms, for example, chromatin remodeling through various histone modification or DNA methylation processes [146]. Such mechanisms allow organisms to respond to the environment through changes in gene expression patterns that are subsequently retained through mitosis [147].

Nevertheless, modeling strategies can already be used to discover molecular events that result from complex circuit behavior. Recently, a systems biology approach in the *Arabidopsis* model system has been used to strongly improve our understanding of the photoperiodic regulation of flowering time [148]. Recent controlled environment studies have included more complex temperature and photoperiod interactions and

perhaps as a result have uncovered more subtle environment-dependent effects of known flowering time genes [149–151]. Together, these studies have highlighted how a combination of field and controlled environment studies can be used to explore genetic determination of phenological traits and their role in adaptation to environment.

In conclusion, we can predict that sophisticated model-assisted strategies will be applicable to crop research. Models that integrate genetic, molecular, and biochemical information will help understand the complex behavior, such as pathway integration. Models that include the molecular, cellular, whole plant, or field level will show immense potential in crop improvement and crop yield prediction.

9.5 The Genomic Revolution

The comparative approach is central to plant genetic research for both gene isolation and identification of gene function. Similarity in gene sequence between model organisms, in which knowledge is more advanced, and crops has been extensively used in order to identify key genes useful for agronomical and qualitative traits.

During the past 40 years, the study of DNA sequences certainly revolutionized plant genetics and breeding, and, more recently, deep genome sequencing is producing massive amount of information on gene sequences, structures, and functions. Thanks to the modern sequencing technologies, the so-called next-generation sequencing (NGS), the deciphering of complete genomes is advancing with an unprecedented pace, challenging scientists to find new high-throughput methods for gene function studies and to develop dense genetic and physical association maps for breeding.

The first plant genome sequencing was completed in 2000 on the model plant *A. thaliana* [152], by implementing an ordered hierarchical strategy that involved the construction of bacterial artificial chromosomes (BAC) libraries, physical mapping, and establishment of a minimum tilling path constituted by the minimum number of partially overlapped BACs that cover the entire genome. The 150 Mbp *Arabidopsis* genome was a relevant milestone that prompted other research groups to run new plant genome projects. After few years, the 398 Mbp rice genome was completed [153]. These first approaches were implemented by using the classical Sanger method that produces high fidelity large reads and can be robustly automated. These achievements demonstrated that with a good organization, human resources, and enough money, complex eukaryotic genomes could be deciphered in a limited time span. However, sequencing work was quite cumbersome, involving hundreds of scientists, and overall costs were quite high. Despite these limiting factors other plant genomes quickly followed, by using variants of the Sanger method, named whole-genome shotgun, which is based on the random sequencing of different genomic libraries of various sizes, in order to have appropriate genome coverage: poplar tree, grapevine, papaya, sorghum, cucumber, maize, soybean, *Brachypodium*,

peach [154–162] (http://www.appliedgenomics.org/news/pressroom/article/2010/apr/02/welcome-peach-genome/; March 1, 2011).

To overcome the limitations of the Sanger method, new technologies that allow sample high throughputs, require no cloning step, and have lower costs, have been rapidly deployed and are now commercially available. These NGS platforms are able to produce large amount of DNA sequence reads (usually millions of bases) of short lengths ranging between 25 and 500 bp (for comprehensive reviews of NGS, see Refs [163–165].

Present plant genome projects are quickly adopting NGS approaches to gain deep insights into chromosomal structures. Recently, apple genome draft assembly has been accomplished by using a combination of Sanger and Roche 454 sequencing (23× and 51× coverage, respectively) [166]. Assembly covered 80% of the 740 Mbp genome.

The same approach was used to compile the genome of *Theobroma cacao*, an economically important tropical fruit-tree crop that is the source of chocolate. The assembly corresponds to 76% of the estimated genome size and contains almost all previously described genes, with 82% of these genes anchored on the 10 *T. cacao* chromosomes [167]. The first application of a complex genome sequence being completed without the help of the Sanger method has been achieved in oil palm genome. In this case, Roche 454 sequencing, using a combination of whole genome and BAC sequencing, was used to produce the 1.7 Gbp genome sequence (http://tinyurl.com/palmgenome; March 1, 2011). A combined Illumina Solexa and Roche 454 sequencing has been used to read the genome of a wild Peruvian cotton species, *Gossypium raimondii* (http://www.monsanto.com/newsviews/Pages/Monsanto-Illumina-Key-Milestone-Cotton-Genome-Sequencing.aspx; March 1, 2011). *De novo* approach with NGS was used to sequence the 240 MBp genome of *Fragaria vesca*, the woodland strawberry, a model plant relative of the cultivated strawberry. The draft *F. vesca*, genome sequenced with a combination of Roche 454, Illumina, and Solid platforms to ×39 coverage was anchored to the genetic linkage map into seven pseudochromosomes [168].

An important effort is taking place in China where the Beijing Genome Institute in Shenzhen (BGI), a high-tech core sequencing facility, in collaboration with other national research institutes, is on the way of sequencing many crop genomes. By using a hybrid strategy that combines 4× Sanger and 68× Illumina Solexa BGI produced more than 96% of the cucumber genome [159]. This achievement was followed by the 50× coverage of the *Brassica rapa* genome by using the Illumina GAII technology.

Several sequencing projects are active at international levels and, given the speed of the sequencing technology developments, it is highly likely that in few years major crop genome sequences will be available to plant scientists and breeders.

The principal advantage of NGS is that their throughputs are much higher than that of classical sequencing. Modern platforms are able to produce more than 100 Gbp compared to the 100 Kb capacity of the Sanger machines [163]. Also, the sequencing cost has dropped dramatically in 10 years from 10,000 $/Mbp to 1 $/Mbp of the last-generation instruments. Although the cost of the NSG technology is now

halving every year, the short reads and the frequent sequencing errors and artifacts pose novel relevant problems in annotation and assembly. Today, the major cost in the production of a complete assembly of a genome sequence lies in the bioinformatics cost, computing power, and in algorithm development and tailoring.

These problems are limiting NGS mostly to resequencing projects, particularly for the discovery of point mutation (SNPs, single-nucleotide polymorphisms) and the development of molecular markers useful for plant breeding. Although it is certainly possible, and sooner than ever before, *de novo* sequencing is still a challenging and expensive effort.

However, it must be stressed that while a complete genome assembly is relevant to address fundamental biological problems, such as the structure and the evolution of plant genomes, for most of the practical implications in plant genetics and breeding, a lot of knowledge can be gathered from not assembled and low-coverage shotgun sequencing by NGS. Using short paired read data from even 1× coverage can help in identifying *in silico* numerous sequences that are similar in relative or model species. By using the sequence, the entire locus can be isolated and characterized by PCR reconstruction approach. By using this straightforward comparative method, new genes and promoters involved in agronomical traits can be isolated in economically important crops. NGS can effectively support the development of molecular markers, directly sequencing the genome of the crop of interest by finding polymorphism that can be associated with genes of interest.

Molecular markers linked to traits of interest can be used in breeding schemes for marker-assisted selection (MAS), speeding up the production of new varieties, allowing breeders to achieve early selection of the trait. SNPs are by far the most prominent markers in plant genetics, thanks to the advancement achieved with NGS (for an exhaustive review, see Ref. [169]). SNP discovery is based on the finding of single-nucleotide differences between sequences. Classically, differences are found either by amplifying time in PCR target DNA regions in different individuals, followed by direct sequencing of fragments, or by using methods that exploit differences in fragment conformation and mobility, such as high-resolution melting (HRM) or single-strand conformation polymorphism (SSCP) analyses. These approaches are time consuming, particularly in genetic mapping studies and population genotyping, where a high number of samples and large screening is required.

The availability of reference genomes in crops allows an efficient *in silico* search for SNP and SSR by resequencing and comparing the diversity in sequences among strains, lines, and genotypes. The large amount of data produced by NGS allows efficient mining of molecular markers [170].

Many plants have large and complex genomes with an abundance of repeated sequences. Many plants are also polyploid. Large genome sizes, an abundance of repeated sequences, and polyploidy typical of many plants present challenges for genome-wide SNP discovery of total genomic DNA using NGS. In fact, making alignment and clustering of short reads generated by NGS platforms is difficult, particularly in the absence of a reference genome sequence. Despite these hurdles, specific strategies can be developed to mine SNPs. For instance, a pipeline platform

was developed and used for genome-wide SNP discovery in *Aegilops tauschii*, the diploid source of the wheat D genome and with a genome size of 4.02 Gb, of which 90% is repetitive sequences. Roughly, 500 000 SNPs were discovered and were dispersed across the entire *A. tauschii* genome [171].

The use of high-density SNPs association maps can be exploited for genome-wide association (GWA) mapping and genomic selection (GS), which will be increasingly adopted for crop improvement and require a large number of genetic markers. By using reduced representation libraries (RRLs), libraries produced by digesting DNA with a common enzyme increasing the coverage probability, from 17 DNA grapevine sample sequence with Illumina Genome Analyzer, 470 000 SNPs were discovered. Among total SNPs, a subset of 9 000 was chosen to design an array able to discriminate genotypes with high efficiency [172].

In a similar work, a total of 7108–25 047 predicted SNPs were discovered in rapeseed using a reduced representation library that was subsequently sequenced by the Illumina sequence-by-synthesis method on the clonal single-molecule array platform. A high-resolution genetic map using 444 recombinant inbred lines was created with 1790 SNP markers [173]. SNPs can also be discovered by interrogating the plant transcriptome. Massively parallel pyrosequencing technology was used to sequence the transcriptomes of shoot apical meristems isolated from two inbred lines of maize using laser capture microdissection (LCM). A computational pipeline that uses the POLYBAYES polymorphism detection system was adapted for 454 ESTs and used to detect SNPs between the two inbred lines. Over 36 000 putative SNPs were detected. Stringent postprocessing reduced this number to $>$7 000 putative SNPs [174]. The exponential development in genome sequencing technology will not only allow whole-genome sequencing to become the standard in marker discovery, mapping, and population genotyping but also help in functional genomics studies by cDNA sequencing to render microarray technology obsolete [164].

9.6
Plant Phenomics: Bridging the Gap between Genomics and Phenotype

In the last decade, we have been immersed in the era of genomics revolution that is radically changing plant biology and breeding. Today, the continuous annotation of plant and crop genomes is providing scientists with an enormous amount of information on genome structure and evolution, with the potential to revolutionize the way new crop varieties are developed in order to fight agriculture and food production problems worldwide. However, digging into genomics and transcriptomics data to extract meaningful information on gene functions requires still intense efforts. The recent fast adoption of NGS technologies is producing daily terabytes of data worldwide that must be analyzed to search for functional meanings. Despite the high throughput in sequence data production, the association of genes with their function is lagging behind. The study of the relationship of specific genes with the measurable characteristics of a plant, that is, the study of the phenotype, is crucial to understand the specific role and function of genes.

The phenotype is a result of the interaction between genes and environmental stimuli. To discover gene function, scientists usually play with these two factors, using mutants of target genes, either naturally available within the genetic diversity of the species or experimentally induced, and growing plants under different specific environmental conditions. Eventually, changes in phenotypes will give clues for the role of the target gene in plant physiological functions or metabolisms.

The genetic strategy involves the generation of knockout, that is, mutants, in which the gene function is disrupted, and gain-of-function mutants, in which the gene is overexpressed by using genetic engineering technologies. The two approaches should give rise to opposite phenotypes, although they often result in no visible phenotypes. On the other hand, the study of phenotype responses to the stressful environment requires the growth of plant under various environmental conditions. In plant breeding, selection schemes are applied in different fields, years, and seasons requiring intense human efforts and often subjective sampling.

The measurement of the phenotype is a time-consuming step characterized by laborious manual sampling and disruptive measurements.

In plant abiotic stress response breeding, reliable phenotyping protocols are extremely important and their poor development has limited the number of success stories available so far [175].

High-throughput plant phenotyping or phenomics, in "omics" term, is evidently becoming the bottleneck in plant biology to close the gap between plant genetics and physiology. This strong demand recently stimulated various research institutions to invest in developing technologies and platforms able to speed up the phenotyping process. The investments started earlier in the private sectors (Cropdesign, Gent, Belgium; Keygene, Wageningen, Holland), and more recently this has been embraced by public research institutions that are developing an international collaboration network (www.plantphenomics.com; March 1, 2011). Large phenotyping platforms are present in Australia, Germany, France, Canada, and Italy, and others are being developed throughout the world [176, 177].

Plant phenomics platforms represent so far an ensemble of technologies that are mainly based on nondestructive image analyses that exploit either the reflectance properties of the incident light on plant tissues or structural and functional features obtained by sophisticated technologies such as MRI and X-ray CT scanning [178, 179]. In a typical robotized experimental lab setup, cameras can be moved to plants [135, 180] or an entire greenhouse can be fully automated with conveyor belts that carry plants to imaging stations. The latter setup is becoming a standard in plant phenomics, being present in several laboratories around the world (CropDesign, Belgium; The Plant Accelerator, Australia; PhenoPhab, Holland; Metapontum Agrobios, Italy; IPK, Germany), and has the advantage of acquiring images from different angles, allowing 3D analyses.

High processing phenotyping is also possible in open field conditions where cameras can be moved over the plants [181] or taking images from long distance [182]. As for other high-throughput technologies, data storage, data management, and high power computing are essential in plant phenomics for the development of a robust approach. A single high-resolution image has the potential to yield a vast set of

phenotypic descriptors, which must be accurately analyzed and meaningful data selected.

Images can be dynamically taken using light at various wavelengths, such as visible (RGB), near-infrared (NIR), and ultraviolet (UV), which will produce reflectance data useful for morphometric and physiological analyses, such as tissue water potential and photosynthesis efficiency. Thermal infrared imaging can be implemented to interrogate plant transpiration rates. All these parameters are particularly relevant to study abiotic stress responses such as drought stress (for detailed review, see Ref. [183]).

A commercially available image capture and analysis system (Lemnatec Scanalyzer 3D) was used to take nondestructive measurements of plant growth and health in 12 *Triticum monococcum* accessions, and the data thereof complemented with chemical and physical analyses, to identify three main components of salinity tolerance [184].

An automated platform for reproducible phenotyping of plant responses to soil water deficit in *A. thaliana* was developed in France [135]. Using this system, nine accessions were grown in four experiments in a rigorously controlled growth chamber equipped with an automated system to control soil water content and take pictures of individual plants.

The simultaneous detection of the reaction of plant growth and chlorophyll fluorescence-related parameters was obtained using a novel approach that combines existing imaging technologies (GROWSCREEN FLUORO). Three different abiotic stress situations were investigated demonstrating the benefit of this approach to distinguish between effects related to (1) growth, (2) chlorophyll-fluorescence, or (3) both of these aspects of the phenotype in *Arabidopsis* and tobacco [180].

Expansive growth of leaves or of reproductive organs, such as silks, is affected by water deficit before any reduction in photosynthesis or root growth. A specific platform, Phenodyn, was set up to perform a genetic analysis of growth and gas exchanges that vary rapidly with environmental conditions in hundreds of maize lines. In particular, authors intended to disentangle the genetic basis of the differences in growth rate and of its responses to temperature, evaporative demand, and soil water deficit [185].

9.7 Conclusions

In the last few decades, extraordinary progress has been made in unraveling the molecular basis of plant responses to the environment. Research from many laboratories has uncovered key regulatory circuits underlying gene expression reprogramming occurring in response to diverse abiotic stresses, to demonstrate the existence of multiple interactions and crosstalks among different signaling pathways, and to identify a core set of multistress responsive genes evolutionarily conserved in all organisms. However, much remains to be done to fully understand the whole stress response system of plants. Major efforts will be needed to expand our knowledge of transcription networks underpinning abiotic stress responses, to

integrate transcriptional control with other levels of regulation, such as noncoding RNAs and chromatin remodeling, to develop experimental settings that mimic the various combinations of environmental stresses occurring in the field. Systems biology approaches will help understand complex plant behavior in a changing environment and will show immense potential in crop improvement and crop yield prediction.

Due to both the complexity of plant stress response mechanisms and the large data sets generated by the "omics" technologies, knowledge transfer into crop breeding still poses big challenges and needs significant efforts in the mining of meaningful information. The characterization of plant phenotypes, useful for variety constitution, represents today the bottleneck of plant biology and the number of known agronomical traits is very low compared to the power of production of NGS and the application potential of genomic sequences.

A bridge between genomics and plant phenotype must be quickly developed in order to fill the gap and meet the increasing world demand for food both in quality and quantity. The recent growth of plant phenomics platforms and initiative at world level indicates that the era of the application of genomics is just starting.

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References

1 Acquaah, G. (2007) *Principles of Plant Genetics and Breeding*, Blackwell, Oxford, UK.
2 Delmer, D.P. (2005) Agriculture in the developing world: connecting innovations in plant research to downstream applications. *Proc. Natl. Acad. Sci. USA*, **102** (44), 15739–15746.
3 Hua, J. (2009) From freezing to scorching: transcriptional responses to temperature variations in plants. *Curr. Opin. Plant Biol.*, **12** (5), 568–573.
4 Thomashow, M.F. (2010) Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiol.*, **154** (2), 571–577.
5 Miura, K., Jin, J.B., Lee, J., Yoo, C.Y., Stirm, V., Miura, T., Ashworth, E.N., Bressan, R.A., Yun, D.J., and Hasegawa, P.M. (2007) SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in *Arabidopsis*. *Plant Cell*, **19** (4), 1403–1414.
6 Doherty, C.J., Van Buskirk, H.A., Myers, S.J., and Thomashow, M.F. (2009) Roles for Arabidopsis CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *Plant Cell*, **21** (3), 972–984.
7 Fowler, S.G., Cook, D., and Thomashow, M.F. (2005) Low

temperature induction of Arabidopsis CBF1, 2, and 3 is gated by the circadian clock. *Plant Physiol.*, **137** (3), 961–968.

8 Franklin, K.A. and Whitelam, G.C. (2007) Light-quality regulation of freezing tolerance in *Arabidopsis thaliana*. *Nat. Genet.*, **39** (11), 1410–1413.

9 Koornneef, M., Alonso-Blanco, C., and Vreugdenhil, D. (2004) Naturally occurring genetic variation in *Arabidopsis thaliana*. *Annu. Rev. Plant Biol.*, **55**, 141–172.

10 Zhen, Y. and Ungerer, M.C. (2008) Relaxed selection on the CBF/DREB1 regulatory genes and reduced freezing tolerance in the southern range of *Arabidopsis thaliana*. *Mol. Biol. Evol.*, **25** (12), 2547–2555.

11 Gilmour, S.J., Fowler, S.G., and Thomashow, M.F. (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol. Biol.*, **54** (5), 767–781.

12 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*, **10** (8), 1391–1406.

13 Achard, P., Gong, F., Cheminant, S., Alioua, M., Hedden, P., and Genschik, P. (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. *Plant Cell*, **20** (8), 2117–2129.

14 Harberd, N.P., Belfield, E., and Yasumura, Y. (2009) The angiosperm gibberellin-GID1-DELLA growth regulatory mechanism: how an "inhibitor of an inhibitor" enables flexible response to fluctuating environments. *Plant Cell*, **21** (5), 1328–1339.

15 Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T., and Thomashow, M.F. (2001) Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.*, **127** (3), 910–917.

16 Nakashima, K., Fujita, Y., Kanamori, N., Katagiri, T., Umezawa, T., Kidokoro, S., Maruyama, K., Yoshida, T., Ishiyama, K., Kobayashi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2009) Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant Cell Physiol.*, **50** (7), 1345–1363.

17 Chew, Y.H. and Halliday, K.J. (2011) A stress-free walk from Arabidopsis to crops. *Curr. Opin. Biotechnol.*, **22** (2), 281–286.

18 Mittler, R. and Blumwald, E. (2010) Genetic engineering for modern agriculture: challenges and perspectives. *Annu. Rev. Plant Biol.*, **61**, 443–462.

19 Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., and Abrams, S.R. (2010) Abscisic acid: emergence of a core signaling network. *Annu. Rev. Plant Biol.*, **61**, 651–679.

20 Sirichandra, C., Wasilewska, A., Vlad, F., Valon, C., and Leung, J. (2009) The guard cell as a single-cell model towards understanding drought tolerance and abscisic acid action. *J. Exp. Bot.*, **60** (5), 1439–1463.

21 Park, S.Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T.F., Alfred, S.E., Bonetta, D., Finkelstein, R., Provart, N.J., Desveaux, D., Rodriguez, P.L., McCourt, P., Zhu, J.K., Schroeder, J.I., Volkman, B.F., and Cutler, S.R. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science*, **324** (5930), 1068–1071.

22 Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., and Grill, E. (2009) Regulators of PP2C phosphatase activity function as abscisic

acid sensors. *Science*, **324** (5930), 1064–1068.

23 Santiago, J., Rodrigues, A., Saez, A., Rubio, S., Antoni, R., Dupeux, F., Park, S.Y., Marquez, J.A., Cutler, S.R., and Rodriguez, P.L. (2009) Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. *Plant J.*, **60** (4), 575–588.

24 Nishimura, N., Sarkeshik, A., Nito, K., Park, S.Y., Wang, A., Carvalho, P.C., Lee, S., Caddell, D.F., Cutler, S.R., Chory, J., Yates, J.R., and Schroeder, J.I. (2010) PYR/PYL/RCAR family members are major *in-vivo* ABI1 protein phosphatase 2C-interacting proteins in *Arabidopsis*. *Plant J.*, **61** (2), 290–299.

25 Hubbard, K.E., Nishimura, N., Hitomi, K., Getzoff, E.D., and Schroeder, J.I. (2010) Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes Dev.*, **24** (16), 1695–1708.

26 Iyer, L.M., Koonin, E.V., and Aravind, L. (2001) Adaptations of the helix-grip fold for ligand binding and catalysis in the START domain superfamily. *Proteins.*, **43** (2), 134–144.

27 Radauer, C., Lackner, P., and Breiteneder, H. (2008) The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evol. Biol.*, **8**, 286.

28 Melcher, K., Ng, L.M., Zhou, X.E., Soon, F.F., Xu, Y., Suino-Powell, K.M., Park, S.Y., Weiner, J.J., Fujii, H., Chinnusamy, V., Kovach, A., Li, J., Wang, Y., Peterson, F.C., Jensen, D.R., Yong, E.L., Volkman, B.F., Cutler, S.R., Zhu, J.K., and Xu, H.E. (2009) A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. *Nature*, **462** (7273), 602–608.

29 Santiago, J., Dupeux, F., Round, A., Antoni, R., Park, S.Y., Jamin, M., Cutler, S.R., Rodriguez, P.L., and Marquez, J.A. (2009) The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature*, **462** (7273), 665–668.

30 Nishimura, N., Hitomi, K., Arvai, A.S., Rambo, R.P., Hitomi, C., Cutler, S.R., Schroeder, J.I., and Getzoff, E.D. (2009) Structural mechanism of abscisic acid binding and signaling by dimeric PYR1. *Science*, **326** (5958), 1373–1379.

31 Yin, P., Fan, H., Hao, Q., Yuan, X., Wu, D., Pang, Y., Yan, C., Li, W., Wang, J., and Yan, N. (2009) Structural insights into the mechanism of abscisic acid signaling by PYL proteins. *Nat. Struct. Mol. Biol.*, **16** (12), 1230–1236.

32 Miyazono, K., Miyakawa, T., Sawano, Y., Kubota, K., Kang, H.J., Asano, A., Miyauchi, Y., Takahashi, M., Zhi, Y., Fujita, Y., Yoshida, T., Kodaira, K.S., Yamaguchi-Shinozaki, K., and Tanokura, M. (2009) Structural basis of abscisic acid signalling. *Nature.*, **462** (7273), 609–614.

33 Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, J. (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell*, **14** (12), 3089–3099.

34 Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F., and Shinozaki, K. (2006) The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. *J. Biol. Chem.*, **281** (8), 5310–5318.

35 Fujii, H. and Zhu, J.K. (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc. Natl. Acad. Sci. USA*, **106** (20), 8380–8385.

36 Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., Umezawa, T., Fujita, M., Maruyama, K., Ishiyama, K., Kobayashi, M., Nakasone, S., Yamada, K., Ito, T., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2009) Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in *Arabidopsis*. *Plant Cell Physiol.*, **50** (12), 2123–2132.

37 Nakashima, K. and Yamaguchi-Shinozaki, K. (2006) Regulons involved

in osmotic stress-responsive and cold stress-responsive gene expression in plants. *Physiol. Plant.*, **126** (1), 62–71.

38 Belin, C., de Franco, P.O., Bourbousse, C., Chaignepain, S., Schmitter, J.M., Vavasseur, A., Giraudat, J., Barbier-Brygoo, H., and Thomine, S. (2006) Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant. Physiol.*, **141** (4), 1316–1327.

39 Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., and Shinozaki, K. (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **106** (41), 17588–17593.

40 Vlad, F., Rubio, S., Rodrigues, A., Sirichandra, C., Belin, C., Robert, N., Leung, J., Rodriguez, P.L., Lauriere, C., and Merlot, S. (2009) Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in *Arabidopsis*. *Plant. Cell.*, **21** (10), 3170–3184.

41 Weiner, J.J., Peterson, F.C., Volkman, B.F., and Cutler, S.R. (2010) Structural and functional insights into core ABA signaling. *Curr. Opin. Plant. Biol.*, **13** (5), 495–502.

42 Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.Y., Cutler, S.R., Sheen, J., Rodriguez, P.L., and Zhu, J.K. (2009) *In vitro* reconstitution of an abscisic acid signalling pathway. *Nature*, **462** (7273), 660–664.

43 Kline, K.G., Sussman, M.R., and Jones, A.M. (2010) Abscisic acid receptors. *Plant. Physiol.*, **154** (2), 479–482.

44 Klingler, J.P., Batelli, G., and Zhu, J.K. (2010) ABA receptors: the START of a new paradigm in phytohormone signalling. *J. Exp. Bot.*, **61** (12), 3199–3210.

45 Melcher, K., Xu, Y., Ng, L.M., Zhou, X.E., Soon, F.F., Chinnusamy, V., Suino-Powell, K.M., Kovach, A., Tham, F.S., Cutler, S.R., Li, J., Yong, E.L., Zhu, J.K., and Xu, H.E. (2010) Identification and mechanism of ABA receptor antagonism. *Nat. Struct. Mol. Biol.*, **17** (9), 1102–1108.

46 Peterson, F.C., Burgie, E.S., Park, S.Y., Jensen, D.R., Weiner, J.J., Bingman, C.A., Chang, C.E., Cutler, S.R., Phillips, G.N., Jr., and Volkman, B.F. (2010) Structural basis for selective activation of ABA receptors. *Nat. Struct. Mol. Biol.*, **17** (9), 1109–1113.

47 Eckardt, N.A., Cominelli, E., Galbiati, M., and Tonelli, C. (2009) The future of science: food and water for life. *Plant Cell*, **21** (2), 368–372.

48 Bartels, D. and Sunkar, R. (2005) Drought and salt tolerance in plants. *Crit. Rev. Plant Sci.*, **24** (1), 23–58.

49 Bartels, D. and Salamini, F. (2001) Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at the molecular level. *Plant Physiol.*, **127** (4), 1346–1353.

50 Ashraf, M. (2010) Inducing drought tolerance in plants: recent advances. *Biotechnol. Adv.*, **28** (1), 169–183.

51 Xoconostle-Cazares, B., Ramirez-Ortega, F.A., Flores-Elenes, L., and Ruiz-Medrano, R. (2010) Drought tolerance in crop plants. *Am. J. Plant Physiol.*, **5** (5), 241–256.

52 Century, K., Reuber, T.L., and Ratcliffe, O.J. (2008) Regulating the regulators: the future prospects for transcription-factor-based agricultural biotechnology products. *Plant Physiol.*, **147** (1), 20–29.

53 Bray, E.A. (1993) Molecular responses to water deficit. *Plant Physiol.*, **103** (4), 1035–1040.

54 Kishor, P., Hong, Z., Miao, G.H., Hu, C., and Verma, D. (1995) Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.*, **108** (4), 1387–1394.

55 Zhu, B., Su, J., Chang, M., Verma, D.P.S., Fan, Y.-L., and Wu, R. (1998) Overexpression of a D1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water- and salt-stress in transgenic rice. *Plant Sci.*, **139** (1), 41–48.

56 Molinari, H.B.C., Marur, C.J., Daros, E., De Campos, M.K.F.,

De Carvalho, J.F.R.P., Filho, J.C.B., Pereira, L.F.P., and Vieira, L.G.E. (2007) Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress. *Physiol. Plant.*, **130** (2), 218–229.

57 Rathinasabapathi, B., McCue, K.F., Gage, D.A., and Hanson, A.D. (1994) Metabolic engineering of glycine betaine synthesis: plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance. *Planta*, **193** (2), 155–162.

58 Sakamoto, A. and Murata, N. (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant Cell Environ.*, **25** (2), 163–171.

59 Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D., and Somero, G.N. (1982) Living with water stress: evolution of osmolyte systems. *Science.*, **217** (4566), 1214–1222.

60 Mattioli, R., Costantino, P., and Trovato, M. (2009) Proline accumulation in plants: not only stress. *Plant. Signal. Behav.*, **4** (11), 1016–1018.

61 Szabados, L. and Savoure, A. (2010) Proline: a multifunctional amino acid. *Trends Plant Sci.*, **15** (2), 89–97.

62 Verbruggen, N. and Hermans, C. (2008) Proline accumulation in plants: a review. *Amino Acids*, **35** (4), 753–759.

63 Hong, Z., Lakkineni, K., Zhang, Z., and Verma, D.P. (2000) Removal of feedback inhibition of delta(1)-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiol.*, **122** (4), 1129–1136.

64 Kumar, V., Varsha, S., Kavi Kishor, P.B., Jawali, N., and Shitole, M.G. (2010) Enhanced proline accumulation and salt stress tolerance of transgenic indica rice by over-expressing P5CSF129A gene. *Plant Biotechnol. Rep.*, **4**, 37–48.

65 Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Lett.*, **461** (3), 205–210.

66 Mani, S., Van De Cotte, B., Van Montagu, M., and Verbruggen, N. (2002) Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in *Arabidopsis*. *Plant Physiol.*, **128** (1), 73–83.

67 Banu, M.N., Hoque, M.A., Watanabe-Sugimoto, M., Islam, M.M., Uraji, M., Matsuoka, K., Nakamura, Y., and Murata, Y. (2010) Proline and glycinebetaine ameliorated NaCl stress via scavenging of hydrogen peroxide and methylglyoxal but not superoxide or nitric oxide in tobacco cultured cells. *Biosci. Biotechnol. Biochem.*, **74** (10), 2043–2049.

68 Papageorgiou, G.C. and Murata, N. (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. *Photosynth. Res.*, **44** (3), 243–252.

69 Rhodes, D. and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **44** (1), 357–384.

70 Nyyssola, A., Kerovuo, J., Kaukinen, P., von Weymarn, N., and Reinikainen, T. (2000) Extreme halophiles synthesize betaine from glycine by methylation. *J. Biol. Chem.*, **275** (29), 22196–22201.

71 Landfald, B. and Strom, A.R. (1986) Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. *J. Bacteriol.*, **165** (3), 849–855.

72 Holmstrom, K.O., Somersalo, S., Mandal, A., Palva, T.E., and Welin, B. (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. *J. Exp. Bot.*, **51** (343), 177–185.

73 Deshnium, P., Los, D.A., Hayashi, H., Mustardy, L., and Murata, N. (1995) Transformation of *Synechococcus* with a gene for choline oxidase enhances tolerance to salt stress. *Plant Mol. Biol.*, **29** (5), 897–907.

74 Sakamoto, A. and Murata, N. (1998) Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. *Plant Mol. Biol.*, **38** (6), 1011–1019.

75 Shirasawa, K., Takabe, T., and Kishitani, S. (2006) Accumulation of glycinebetaine in rice plants that overexpress choline monooxygenase from spinach and evaluation of their tolerance to abiotic stress. *Ann. Bot.*, **98** (3), 565–571.

76 McNeil, S.D., Nuccio, M.L., Ziemak, M.J., and Hanson, A.D. (2001) Enhanced synthesis of choline and glycine betaine in transgenic tobacco plants that overexpress phosphoethanolamine *N*-methyltransferase. *Proc. Natl. Acad. Sci. USA*, **98** (17), 10001–10005.

77 Quan, R., Shang, M., Zhang, H., Zhao, Y., and Zhang, J. (2004) Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. *Plant Biotechnol. J*, **2** (6), 477–486.

78 Quan, R., Shang, M., Zhang, H., Zhao, Y., and Juren, Z. (2004) Improved chilling tolerance by transformation with betA gene for the enhancement of glycinebetaine synthesis in maize. *Plant Sci.*, **166** (1), 141–149.

79 Park, E.J., Jeknic, Z., Pino, M.T., Murata, N., and Chen, T.H. (2007) Glycinebetaine accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic tomato plants against abiotic stress. *Plant Cell Environ.*, **30** (8), 994–1005.

80 Doebley, J.F., Gaut, B.S., and Smith, B.D. (2006) The molecular genetics of crop domestication. *Cell*, **127** (7), 1309–1321.

81 Yu, H., Chen, X., Hong, Y.Y., Wang, Y., Xu, P., Ke, S.D., Liu, H.Y., Zhu, J.K., Oliver, D.J., and Xiang, C.B. (2008) Activated expression of an Arabidopsis HD-START protein confers drought tolerance with improved root system and reduced stomatal density. *Plant Cell*, **20** (4), 1134–1151.

82 Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science*, **280** (5360), 104–106.

83 Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D., and Thomashow, M.F. (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.*, **124** (4), 1854–1865.

84 Ding, Z., Li, S., An, X., Liu, X., Qin, H., and Wang, D. (2009) Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*. *J. Genet. Genomics*, **36** (1), 17–29.

85 Jung, C., Seo, J.S., Han, S.W., Koo, Y.J., Kim, C.H., Song, S.I., Nahm, B.H., Choi, Y.D., and Cheong, J.J. (2008) Overexpression of AtMYB44 enhances stomatal closure to confer abiotic stress tolerance in transgenic *Arabidopsis*. *Plant Physiol.*, **146** (2), 623–635.

86 Wu, Y., Deng, Z., Lai, J., Zhang, Y., Yang, C., Yin, B., Zhao, Q., Zhang, L., Li, Y., and Xie, Q. (2009) Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. *Cell Res.*, **19** (11), 1279–1290.

87 Hsieh, T.H., Lee, J.T., Charng, Y.Y., and Chan, M.T. (2002) Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. *Plant Physiol.*, **130** (2), 618–626.

88 Oh, S.J., Song, S.I., Kim, Y.S., Jang, H.J., Kim, S.Y., Kim, M., Kim, Y.K., Nahm, B.H., and Kim, J.K. (2005) Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiol.*, **138** (1), 341–351.

89 Hsieh, T.H., Lee, J.T., Yang, P.T., Chiu, L.H., Charng, Y.Y., Wang, Y.C., and Chan, M.T. (2002) Heterology expression of the Arabidopsis C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. *Plant Physiol.*, **129** (3), 1086–1094.

90 Yang, J.S., Wang, R., Meng, J.J., Bi, Y.P., Xu, P.L., Guo, F., Wan, S.B., He, Q.W., and Li, X.G. (2010) Overexpression of Arabidopsis CBF1 gene in transgenic tobacco alleviates photoinhibition of PSII

and PSI during chilling stress under low irradiance. *J. Plant Physiol.*, **167** (7), 534–539.

91 Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003) OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J.*, **33** (4), 751–763.

92 Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol.*, **47** (1), 141–153.

93 Orellana, S., Yanez, M., Espinoza, A., Verdugo, I., Gonzalez, E., Ruiz-Lara, S., and Casaretto, J.A. (2010) The transcription factor SlAREB1 confers drought, salt stress tolerance and regulates biotic and abiotic stress-related genes in tomato. *Plant Cell Environ.*, **33** (12), 2191–2208.

94 Hsieh, T.H., Li, C.W., Su, R.C., Cheng, C.P., Sanjaya, Tsai, Y.C., and Chan, M.T. (2010) A tomato bZIP transcription factor, SlAREB, is involved in water deficit and salt stress response. *Planta*, **231** (6), 1459–1473.

95 Navarro, M., Ayax, C., Martinez, Y., Laur, J., El Kayal, W., Marque, C., and Teulieres, C. (2011) Two EguCBF1 genes overexpressed in Eucalyptus display a different impact on stress tolerance and plant development. *Plant Biotechnol. J.*, **9** (1), 50–63.

96 Kang, J.Y., Choi, H.I., Im, M.Y., and Kim, S.Y. (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell.*, **14** (2), 343–357.

97 Oh, S.J., Kwon, C.W., Choi, D.W., Song, S.I., and Kim, J.K. (2007) Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice. *Plant Biotechnol. J.*, **5** (5), 646–656.

98 Kiang, J.G. and Tsokos, G.C. (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol. Ther.*, **80** (2), 183–201.

99 Wu, X., Shiroto, Y., Kishitani, S., Ito, Y., and Toriyama, K. (2009) Enhanced heat and drought tolerance in transgenic rice seedlings overexpressing OsWRKY11 under the control of HSP101 promoter. *Plant Cell Rep.*, **28** (1), 21–30.

100 Raho, G., Lupotto, E., Hartings, H., Della Torre A., Perrotta, C., and Marmiroli, N. (1996) Tissue-10Hvhsp17 gene promoter in transgenic tobacco plants. *J. Exp. Bot.*, **47** (10), 1587–1594.

101 Kasuga, M., Miura, S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004) A combination of the Arabidopsis DREB1A gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.*, **45** (3), 346–350.

102 Lee, J.T., Prasad, V., Yang, P.T., Wu, J.F., David Ho, T.H., Charng, Y.Y., and Chan, M.T. (2003) Expression of Arabidopsis CBF1 regulated by an ABA/stress inducible promoter in transgenic tomato confers stress tolerance without affecting yield. *Plant Cell Environ.*, **26** (7), 1181–1190.

103 Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. *Curr. Opin. Biotechnol.*, **17** (2), 113–122.

104 Jakab, G., Ton, J., Flors, V., Zimmerli, L., Métraux, J.-P., and Mauch-Mani, B. (2005) Enhancing *Arabidopsis* salt and drought stress tolerance by chemical priming for its abscisic acid responses. *Plant Physiol.*, **139** (1), 267–274.

105 Srivastava, A.K., Ramaswamy, N.K., Suprasanna, P., and D'Souza, S.F. (2010) Genome-wide analysis of thiourea-modulated salinity stress-responsive transcripts in seeds of *Brassica juncea*: identification of signalling and effector components of stress tolerance. *Ann. Bot.*, **106** (5), 663–674.

106 Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M. (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a

repression domain, in *Arabidopsis*. *Plant J.*, **34** (5), 733–739.

107 Staudt, A.C. and Wenkel, S. (2011) Regulation of protein function by "microproteins". *EMBO Rep.*, **12** (1), 35–42.

108 Minoia, S., Petrozza, A., D'Onofrio, O., Piron, F., Mosca, G., Sozio, G., Cellini, F., Bendahmane, A., and Carriero, F. (2010) A new mutant genetic resource for tomato crop improvement by TILLING technology. *BMC Res. Notes*, **3**, 69.

109 Kariola, T., Brader, G., Helenius, E., Li, J., Heino, P., and Palva, E.T. (2006) Early responsive to dehydration 15, a negative regulator of abscisic acid responses in *Arabidopsis*. *Plant Physiol.*, **142** (4), 1559–1573.

110 Alexandre, C., Moller-Steinbach, Y., Schonrock, N., Gruissem, W., and Hennig, L. (2009) Arabidopsis MSI1 is required for negative regulation of the response to drought stress. *Mol. Plant*, **2** (4), 675–687.

111 Cominelli, E., Galbiati, M., Vavasseur, A., Conti, L., Sala, T., Vuylsteke, M., Leonhardt, N., Dellaporta, S.L., and Tonelli, C. (2005) A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. *Curr. Biol.*, **15** (13), 1196–1200.

112 Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., and Xiong, L. (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci. USA*, **103** (35), 12987–12992.

113 Huang, X.Y., Chao, D.Y., Gao, J.P., Zhu, M.Z., Shi, M., and Lin, H.X. (2009) A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. *Genes Dev.*, **23** (15), 1805–1817.

114 Covarrubias, A.A. and Reyes, J.L. (2010) Post-transcriptional gene regulation of salinity and drought responses by plant microRNAs. *Plant Cell Environ.*, **33** (4), 481–489.

115 Bohnert, H., Gong, Q., Li, P., and Ma, S. (2006) Unraveling abiotic stress tolerance mechanisms – getting genomics going. *Curr. Opin. Plant Biol.*, **9** (2), 180–188.

116 De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A., Pozo, M.J., Mueller, M.J., Buchala, A.J., Métraux, J.-P., Van Loon, L.C., Dicke, M., and Pieterse, C.M.J. (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol. Plant Microbe Interact.*, **18** (9), 923–937.

117 Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr. Opin. Plant Biol.*, **9** (4), 436–442.

118 Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., and Harter, K. (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J.*, **50** (2), 347–363.

119 Ma, S. and Bohnert, H.J. (2007) Integration of *Arabidopsis thaliana* stress-related transcript profiles, promoter structures, and cell-specific expression. *Genome Biol.*, **8** (4), R49.

120 Walley, J.W., Coughlan, S., Hudson, M.E., Covington, M.F., Kaspi, R., Banu, G., Harmer, S.L., and Dehesh, K. (2007) Mechanical stress induces biotic and abiotic stress responses via a novel *cis*-element. *PLoS Genet.*, **3** (10), 1800–1812.

121 Kultz, D. (2005) Molecular and evolutionary basis of the cellular stress response. *Annu. Rev. Physiol.*, **67**, 225–257.

122 Mittler, R. (2006) Abiotic stress, the field environment and stress combination. *Trends Plant Sci.*, **11** (1), 15–19.

123 Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T., and Luan, S. (2002) Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in *Arabidopsis*. *Plant Physiol.*, **129** (2), 661–677.

124 Fowler, S. and Thomashow, M.F. (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell.*, **14** (8), 1675–1690.

125 Rizhsky, L., Liang, H., and Mittler, R. (2002) The combined effect of drought stress and heat shock on gene expression in tobacco. *Plant Physiol.*, **130** (3), 1143–1151.

126 Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., and Mittler, R. (2004) When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. *Plant Physiol.*, **134** (4), 1683–1696.

127 Larkindale, J. and Huang, B. (2004) Thermotolerance and antioxidant systems in *Agrostis stolonifera*: involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene. *J. Plant Physiol.*, **161** (4), 405–413.

128 Hewezi, T., Leger, M., and Gentzbittel, L. (2008) A comprehensive analysis of the combined effects of high light and high temperature stresses on gene expression in sunflower. *Ann. Bot.*, **102** (1), 127–140.

129 Keles, Y. and Öncel, I. (2002) Response of antioxidative defence system to temperature and water stress combinations in wheat seedlings. *Plant Sci.*, **163** (4), 783–790.

130 Hirayama, T. and Shinozaki, K. (2010) Research on plant abiotic stress responses in the post-genome era: past, present and future. *Plant J.*, **61** (6), 1041–1052.

131 Munns, R. and Tester, M. (2008) Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.*, **59**, 651–681.

132 Werner, T., Nehnevajova, E., Kollmer, I., Novak, O., Strnad, M., Kramer, U., and Schmulling, T. (2010) Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. *Plant Cell*, **22** (12), 3905–3920.

133 Yuan, J.S., Galbraith, D.W., Dai, S.Y., Griffin, P., and Stewart, C.N. (2008) Plant systems biology comes of age. *Trends Plant Sci.*, **13** (4), 165–171.

134 Chenu, K., Chapman, S.C., Tardieu, F., McLean, G., Welcker, C., and Hammer, G.L. (2009) Simulating the yield impacts of organ-level quantitative trait loci associated with drought response in maize: a "gene-to-phenotype" modeling approach. *Genetics*, **183** (4), 1507–1523.

135 Granier, C., Aguirrezabal, L., Chenu, K., Cookson, S.J., Dauzat, M., Hamard, P., Thioux, J.J., Rolland, G., Bouchier-Combaud, S., Lebaudy, A., Muller, B., Simonneau, T., and Tardieu, F. (2006) Phenopsis: an automated platform for reproducible phenotyping of plant responses to soil water deficit in *Arabidopsis thaliana* permitted the identification of an accession with low sensitivity to soil water deficit. *New Phytol.*, **169** (3), 623–635.

136 Hammer, G., Cooper, M., Tardieu, F., Welch, S., Walsh, B., van Eeuwijk, F., Chapman, S., and Podlich, D. (2006) Models for navigating biological complexity in breeding improved crop plants. *Trends Plant Sci.*, **11** (12), 587–593.

137 Poorter, H., Niinemets, U., Walter, A., Fiorani, F., and Schurr, U. (2010) A method to construct dose-response curves for a wide range of environmental factors and plant traits by means of a meta-analysis of phenotypic data. *J. Exp. Bot.*, **61** (8), 2043–2055.

138 Tardieu, F. and Tuberosa, R. (2010) Dissection and modelling of abiotic stress tolerance in plants. *Curr. Opin. Plant Biol.*, **13** (2), 206–212.

139 Eckardt, N.A., Araki, T., Benning, C., Cubas, P., Goodrich, J., Jacobsen, S.E., Masson, P., Nambara, E., Simon, R., Somerville, S., and Wasteneys, G. (2001) Arabidopsis research 2001. *Plant Cell.*, **13** (9), 1973–1982.

140 Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T., and Yamanishi, Y. (2008) KEGG for linking genomes to life and the environment. *Nucleic Acids Res.*, **36** (Database issue), D480–D484

141 Matsuda, F., Hirai, M.Y., Sasaki, E., Akiyama, K., Yonekura-Sakakibara, K., Provart, N.J., Sakurai, T., Shimada, Y., and Saito, K. (2010) AtMetExpress development: a phytochemical atlas of *Arabidopsis* development. *Plant Physiol.*, **152** (2), 566–578.

142 Zhang, P., Foerster, H., Tissier, C.P., Mueller, L., Paley, S., Karp, P.D., and Rhee, S.Y. (2005) MetaCyc and AraCyc. Metabolic pathway databases for plant research. *Plant Physiol.*, **138** (1), 27–37.

143 Matsui, A., Ishida, J., Morosawa, T., Mochizuki, Y., Kaminuma, E., Endo, T.A., Okamoto, M., Nambara, E., Nakajima, M., Kawashima, M., Satou, M., Kim, J.M., Kobayashi, N., Toyoda, T., Shinozaki, K., and Seki, M. (2008) Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant Cell Physiol.*, **49** (8), 1135–1149.

144 Matzke, M., Kanno, T., Huettel, B., Daxinger, L., and Matzke, A.J. (2007) Targets of RNA-directed DNA methylation. *Curr. Opin. Plant Biol.*, **10** (5), 512–519.

145 Zeller, G., Henz, S.R., Widmer, C.K., Sachsenberg, T., Ratsch, G., Weigel, D., and Laubinger, S. (2009) Stress-induced changes in the *Arabidopsis thaliana* transcriptome analyzed using whole-genome tiling arrays. *Plant J.*, **58** (6), 1068–1082.

146 Chinnusamy, V. and Zhu, J.K. (2009) Epigenetic regulation of stress responses in plants. *Curr. Opin. Plant Biol.*, **12** (2), 133–139.

147 Jaenisch, R. and Bird, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33** (Suppl.), 245–254.

148 Salazar, J.D., Saithong, T., Brown, P.E., Foreman, J., Locke, J.C.W., Halliday, K.J., Carré, I.A., Rand, D.A., and Millar, A.J. (2009) Prediction of photoperiodic regulators from quantitative gene circuit models. *Cell*, **139** (6), 1170–1179.

149 Li, Y., Roycewicz, P., Smith, E., and Borevitz, J.O. (2006) Genetics of local adaptation in the laboratory: flowering time quantitative trait loci under geographic and seasonal conditions in *Arabidopsis*. *PLoS one*, **1** (1), e105.

150 Scarcelli, N., Cheverud, J.M., Schaal, B.A., and Kover, P.X. (2007) Antagonistic pleiotropic effects reduce the potential adaptive value of the FRIGIDA locus. *Proc. Natl. Acad. Sci. USA*, **104** (43), 16986–16991.

151 Scarcelli, N. and Kover, P.X. (2009) Standing genetic variation in FRIGIDA mediates experimental evolution of flowering time in *Arabidopsis*. *Mol. Ecol.*, **18** (9), 2039–2049.

152 The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature*, **408** (6814), 796–815.

153 Project, I.R.G.S. (2005) The map-based sequence of the rice genome. *Nature*, **436** (7052), 793–800.

154 Tuskan, G.A., Difazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S., Salamov, A., Schein, J., Sterck, L., Aerts, A., Bhalerao, R.R., Bhalerao, R.P., Blaudez, D., Boerjan, W., Brun, A., Brunner, A., Busov, V., Campbell, M., Carlson, J., Chalot, M., Chapman, J., Chen, G.L., Cooper, D., Coutinho, P.M., Couturier, J., Covert, S., Cronk, Q., Cunningham, R., Davis, J., Degroeve, S., Dejardin, A., Depamphilis, C., Detter, J., Dirks, B., Dubchak, I., Duplessis, S., Ehlting, J., Ellis, B., Gendler, K., Goodstein, D., Gribskov, M., Grimwood, J., Groover, A., Gunter, L., Hamberger, B., Heinze, B., Helariutta, Y., Henrissat, B., Holligan, D., Holt, R., Huang, W., Islam-Faridi, N., Jones, S., Jones-Rhoades, M., Jorgensen, R., Joshi, C., Kangasjarvi, J., Karlsson, J., Kelleher, C., Kirkpatrick, R., Kirst, M., Kohler, A., Kalluri, U., Larimer, F., Leebens-Mack, J., Leple, J.C., Locascio, P., Lou, Y., Lucas, S., Martin, F., Montanini, B., Napoli, C., Nelson, D.R., Nelson, C., Nieminen, K., Nilsson, O., Pereda, V., Peter, G., Philippe, R., Pilate, G., Poliakov, A., Razumovskaya, J., Richardson, P., Rinaldi, C., Ritland, K., Rouze, P., Ryaboy, D., Schmutz, J., Schrader, J., Segerman, B., Shin, H.,

Siddiqui, A., Sterky, F., Terry, A., Tsai, C.J., Uberbacher, E., Unneberg, P., Vahala, J., Wall, K., Wessler, S., Yang, G., Yin, T., Douglas, C., Marra, M., Sandberg, G. Van de Peer, Y., and Rokhsar, D. (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science*, **313** (5793), 1596–1604.

155 Jaillon, O., Aury, J.M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., Choisne, N., Aubourg, S., Vitulo, N., Jubin, C., Vezzi, A., Legeai, F., Hugueney, P., Dasilva, C., Horner, D., Mica, E., Jublot, D., Poulain, J., Bruyere, C., Billault, A., Segurens, B., Gouyvenoux, M., Ugarte, E., Cattonaro, F., Anthouard, V., Vico, V., Del Fabbro, C., Alaux, M., Di Gaspero, G., Dumas, V., Felice, N., Paillard, S., Juman, I., Moroldo, M., Scalabrin, S., Canaguier, A., Le Clainche, I., Malacrida, G., Durand, E., Pesole, G., Laucou, V., Chatelet, P., Merdinoglu, D., Delledonne, M., Pezzotti, M., Lecharny, A., Scarpelli, C., Artiguenave, F., Pe, M.E., Valle, G., Morgante, M., Caboche, M., Adam-Blondon, A.F., Weissenbach, J., Quetier, F., and Wincker, P. (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature*, **449** (7161), 463–467.

156 Zharkikh, A., Troggio, M., Pruss, D., Cestaro, A., Eldrdge, G., Pindo, M., Mitchell, J.T., Vezzulli, S., Bhatnagar, S., Fontana, P., Viola, R., Gutin, A., Salamini, F., Skolnick, M., and Velasco, R. (2008) Sequencing and assembly of highly heterozygous genome of *Vitis vinifera* L. cv Pinot Noir: problems and solutions. *J. Biotechnol.*, **136** (1–2), 38–43.

157 Ming, R., Hou, S., Feng, Y., Yu, Q., Dionne-Laporte, A., Saw, J.H., Senin, P., Wang, W., Ly, B.V., Lewis, K.L., Salzberg, S.L., Feng, L., Jones, M.R., Skelton, R.L., Murray, J.E., Chen, C., Qian, W., Shen, J., Du, P., Eustice, M., Tong, E., Tang, H., Lyons, E., Paull, R.E., Michael, T.P., Wall, K., Rice, D.W., Albert, H., Wang, M.L., Zhu, Y.J., Schatz, M., Nagarajan, N., Acob, R.A., Guan, P., Blas, A., Wai, C.M., Ackerman, C.M., Ren, Y., Liu, C., Wang, J., Na, J.K., Shakirov, E.V., Haas, B., Thimmapuram, J., Nelson, D., Wang, X., Bowers, J.E., Gschwend, A.R., Delcher, A.L., Singh, R., Suzuki, J.Y., Tripathi, S., Neupane, K., Wei, H., Irikura, B., Paidi, M., Jiang, N., Zhang, W., Presting, G., Windsor, A., Navajas-Perez, R., Torres, M.J., Feltus, F.A., Porter, B., Li, Y., Burroughs, A.M., Luo, M.C., Liu, L., Christopher, D.A., Mount, S.M., Moore, P.H., Sugimura, T., Jiang, J., Schuler, M.A., Friedman, V., Mitchell-Olds, T., Shippen, D.E., dePamphilis, C.W., Palmer, J.D., Freeling, M., Paterson, A.H., Gonsalves, D., Wang, L., and Alam, M. (2008) The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). *Nature*, **452** (7190), 991–996.

158 Paterson, A.H., Bowers, J.E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, G., Hellsten, U., Mitros, T., Poliakov, A., Schmutz, J., Spannagl, M., Tang, H., Wang, X., Wicker, T., Bharti, A.K., Chapman, J., Feltus, F.A., Gowik, U., Grigoriev, I.V., Lyons, E., Maher, C.A., Martis, M., Narechania, A., Otillar, R.P., Penning, B.W., Salamov, A.A., Wang, Y., Zhang, L., Carpita, N.C., Freeling, M., Gingle, A.R., Hash, C.T., Keller, B., Klein, P., Kresovich, S., McCann, M.C., Ming, R., Peterson, D.G., Mehboob ur, R., Ware, D., Westhoff, P., Mayer, K.F., Messing, J., and Rokhsar, D.S. (2009) The sorghum bicolor genome and the diversification of grasses. *Nature*, **457** (7229), 551–556.

159 Huang, S., Li, R., Zhang, Z., Li, L., Gu, X., Fan, W., Lucas, W.J., Wang, X., Xie, B., Ni, P., Ren, Y., Zhu, H., Li, J., Lin, K., Jin, W., Fei, Z., Li, G., Staub, J., Kilian, A., van der Vossen, E.A., Wu, Y., Guo, J., He, J., Jia, Z., Tian, G., Lu, Y., Ruan, J., Qian, W., Wang, M., Huang, Q., Li, B., Xuan, Z., Cao, J., Asan, Wu, Z., Zhang, J., Cai, Q., Bai, Y., Zhao, B., Han, Y., Li, Y., Li, X., Wang, S., Shi, Q., Liu, S., Cho, W.K., Kim, J.Y., Xu, Y., Heller-Uszynska, K., Miao, H., Cheng, Z., Zhang, S., Wu, J.,

Yang, Y., Kang, H., Li, M., Liang, H., Ren, X., Shi, Z., Wen, M., Jian, M., Yang, H., Zhang, G., Yang, Z., Chen, R., Ma, L., Liu, H., Zhou, Y., Zhao, J., Fang, X., Fang, L., Liu, D., Zheng, H., Zhang, Y., Qin, N., Li, Z., Yang, G., Yang, S., Bolund, L., Kristiansen, K., Li, S., Zhang, X., Wang, J., Sun, R. Zhang, B., Jiang, S., and Du, Y. (2009) The genome of the cucumber, *Cucumis sativus* L. *Nat. Genet.*, **41** (12), 1275–1281.

160 Schnable, P.S., Ware, D., Fulton, R.S., Stein, J.C., Wei, F., Pasternak, S., Liang, C., Zhang, J., Fulton, L., Graves, T.A., Minx, P., Reily, A.D., Courtney, L., Kruchowski, S.S., Tomlinson, C., Strong, C., Delehaunty, K., Fronick, C., Courtney, B., Rock, S.M., Belter, E., Du, F., Kim, K., Abbott, R.M., Cotton, M., Levy, A., Marchetto, P., Ochoa, K., Jackson, S.M., Gillam, B., Chen, W., Yan, L., Higginbotham, J., Cardenas, M., Waligorski, J., Applebaum, E., Phelps, L., Falcone, J., Kanchi, K., Thane, T., Scimone, A., Thane, N., Henke, J., Wang, T., Ruppert, J., Shah, N., Rotter, K., Hodges, J., Ingenthron, E., Cordes, M., Kohlberg, S., Sgro, J., Delgado, B., Mead, K., Chinwalla, A., Leonard, S., Crouse, K., Collura, K., Kudrna, D., Currie, J., He, R., Angelova, A., Rajasekar, S., Mueller, T., Lomeli, R., Scara, G., Ko, A., Delaney, K., Wissotski, M., Lopez, G., Campos, D., Braidotti, M., Ashley, E., Golser, W., Kim, H., Lee, S., Lin, J., Dujmic, Z., Kim, W., Talag, J., Zuccolo, A., Fan, C., Sebastian, A., Kramer, M., Spiegel, L., Nascimento, L., Zutavern, T., Miller, B., Ambroise, C., Muller, S., Spooner, W., Narechania, A., Ren, L., Wei, S., Kumari, S., Faga, B., Levy, M.J., McMahan, L., Van Buren, P., Vaughn, M.W., Ying, K., Yeh, C.T., Emrich, S.J., Jia, Y., Kalyanaraman, A., Hsia, A.P., Barbazuk, W.B., Baucom, R.S., Brutnell, T.P., Carpita, N.C., Chaparro, C., Chia, J.M., Deragon, J.M., Estill, J.C., Fu, Y., Jeddeloh, J.A., Han, Y., Lee, H., Li, P., Lisch, D.R., Liu, S., Liu, Z., Nagel, D.H., McCann, M.C., SanMiguel, P., Myers, A.M., Nettleton, D., Nguyen, J., Penning, B.W., Ponnala, L., Schneider, K.L., Schwartz, D.C., Sharma, A., Soderlund, C., Springer, N.M., Sun, Q., Wang, H., Waterman, M., Westerman, R., Wolfgruber, T.K., Yang, L., Yu, Y., Zhang, L., Zhou, S., Zhu, Q., Bennetzen, J.L., Dawe, R.K., Jiang, J., Jiang, N., Presting, G.G., Wessler, S.R., Aluru, S., Martienssen, R.A., Clifton, S.W., McCombie, W.R., Wing, R.A., and Wilson, R.K. (2009) The B73 maize genome: complexity, diversity, and dynamics. *Science*, **326** (5956), 1112–1115.

161 Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J., Xu, D., Hellsten, U., May, G.D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M.K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X.C., Shinozaki, K., Nguyen, H.T., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C., and Jackson, S.A. (2010) Genome sequence of the palaeopolyploid soybean. *Nature*, **463** (7278), 178–183.

162 Initiative, T.I.B. (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature*, **463** (7282), 763–768.

163 Delseny, M., Han, B., and Hsing, Y.I. (2010) High throughput DNA sequencing: the new sequencing revolution. *Plant Sci.*, **179** (5), 407–422.

164 Edwards, D. and Batley, J. (2010) Plant genome sequencing: applications for crop improvement. *Plant Biotechnol J.*, **8** (1), 2–9.

165 Varshney, R.K., Nayak, S.N., May, G.D., and Jackson, S.A. (2009) Next-generation sequencing technologies and their implications for crop genetics and breeding. *Trends Biotechnol.*, **27** (9), 522–530.

166 Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., Fontana, P., Bhatnagar, S.K., Troggio, M., Pruss, D.,

Salvi, S., Pindo, M., Baldi, P., Castelletti, S., Cavaiuolo, M., Coppola, G., Costa, F., Cova, V., Dal Ri, A., Goremykin, V., Komjanc, M., Longhi, S., Magnago, P., Malacarne, G., Malnoy, M., Micheletti, D., Moretto, M., Perazzolli, M., Si-Ammour, A., Vezzulli, S., Zini, E., Eldredge, G., Fitzgerald, L.M., Gutin, N., Lanchbury, J., Macalma, T., Mitchell, J.T., Reid, J., Wardell, B., Kodira, C., Chen, Z., Desany, B., Niazi, F., Palmer, M., Koepke, T., Jiwan, D., Schaeffer, S., Krishnan, V., Wu, C., Chu, V.T., King, S.T., Vick, J., Tao, Q., Mraz, A., Stormo, A., Stormo, K., Bogden, R., Ederle, D., Stella, A., Vecchietti, A., Kater, M.M., Masiero, S., Lasserre, P., Lespinasse, Y., Allan, A.C., Bus, V., Chagne, D., Crowhurst, R.N., Gleave, A.P., Lavezzo, E., Fawcett, J.A., Proost, S., Rouze, P., Sterck, L., Toppo, S., Lazzari, B., Hellens, R.P., Durel, C.E., Gutin, A., Bumgarner, R.E., Gardiner, S.E., Skolnick, M., Egholm, M., Van de Peer, Y., Salamini, F., and Viola, R. (2010) The genome of the domesticated apple (*Malus x domestica* Borkh). *Nat. Genet.*, **42** (10), 833–839.

167 Argout, X., Salse, J., Aury, J.M., Guiltinan, M.J., Droc, G., Gouzy, J., Allegre, M., Chaparro, C., Legavre, T., Maximova, S.N., Abrouk, M., Murat, F., Fouet, O., Poulain, J., Ruiz, M., Roguet, Y., Rodier-Goud, M., Barbosa-Neto, J.F., Sabot, F., Kudrna, D., Ammiraju, J.S., Schuster, S.C., Carlson, J.E., Sallet, E., Schiex, T., Dievart, A., Kramer, M., Gelley, L., Shi, Z., Berard, A., Viot, C., Boccara, M., Risterucci, A.M., Guignon, V., Sabau, X., Axtell, M.J., Ma, Z., Zhang, Y., Brown, S., Bourge, M., Golser, W., Song, X., Clement, D., Rivallan, R., Tahi, M., Akaza, J.M., Pitollat, B., Gramacho, K., D'Hont, A., Brunel, D., Infante, D., Kebe, I., Costet, P., Wing, R., McCombie, W.R., Guiderdoni, E., Quetier, F., Panaud, O., Wincker, P., Bocs, S., and Lanaud, C. (2011) The genome of *Theobroma cacao*. *Nat. Genet.*, **43** (2), 101–108.

168 Shulaev, V., Sargent, D.J., Crowhurst, R.N., Mockler, T.C., Folkerts, O., Delcher, A.L., Jaiswal, P., Mockaitis, K., Liston, A., Mane, S.P., Burns, P., Davis, T.M., Slovin, J.P., Bassil, N., Hellens, R.P., Evans, C., Harkins, T., Kodira, C., Desany, B., Crasta, O.R., Jensen, R.V., Allan, A.C., Michael, T.P., Setubal, J.C., Celton, J.M., Rees, D.J., Williams, K.P., Holt, S.H., Rojas, J.J., Chatterjee, M., Liu, B., Silva, H., Meisel, L., Adato, A., Filichkin, S.A., Troggio, M., Viola, R., Ashman, T.L., Wang, H., Dharmawardhana, P., Elser, J., Raja, R., Priest, H.D., Bryant, D.W. Jr., Fox, S.E., Givan, S.A., Wilhelm, L.J., Naithani, S., Christoffels, A., Salama, D.Y., Carter, J., Girona, E.L., Zdepski, A., Wang, W., Kerstetter, R.A., Schwab, W., Korban, S.S., Davik, J., Monfort, A., Denoyes-Rothan, B., Arus, P., Mittler, R., Flinn, B., Aharoni, A., Bennetzen, J.L., Salzberg, S.L., Dickerman, A.W., Velasco, R., Borodovsky, M., Veilleux, R.E., and Folta, K.M. (2010) The genome of woodland strawberry (*Fragaria vesca*). *Nat. Genet.*, **43**, 109–116.

169 Ganal, M.W., Altmann, T., and Roder, M.S. (2009) SNP identification in crop plants. *Curr. Opin. Plant Biol.*, **12** (2), 211–217.

170 Imelfort, M., Duran, C., Batley, J., and Edwards, D. (2009) Discovering genetic polymorphisms in next-generation sequencing data. *Plant Biotechnol. J.*, **7** (4), 312–317.

171 You, F.M., Huo, N., Deal, K.R., Gu, Y.Q., Luo, M.C., McGuire, P.E., Dvorak, J., and Anderson, O.D. (2011) Annotation-based genome-wide SNP discovery in the large and complex *Aegilops tauschii* genome using next-generation sequencing without a reference genome sequence. *BMC Genomics*, **12** (1), 59.

172 Myles, S., Chia, J.M., Hurwitz, B., Simon, C., Zhong, G.Y., Buckler, E., and Ware, D. (2010) Rapid genomic characterization of the genus *Vitis*. *PLoS One*, **5** (1), e8219.

173 Hyten, D.L., Cannon, S.B., Song, Q., Weeks, N., Fickus, E.W., Shoemaker, R.C., Specht, J.E.,

Farmer, A.D., May, G.D., and Cregan, P.B. (2010) High-throughput SNP discovery through deep resequencing of a reduced representation library to anchor and orient scaffolds in the soybean whole genome sequence. *BMC Genomics*, **11**, 38.

174 Barbazuk, W.B., Emrich, S.J., Chen, H.D., Li, L., and Schnable, P.S. (2007) SNP discovery via 454 transcriptome sequencing. *Plant J.*, **51** (5), 910–918.

175 Salekdeh, G.H., Reynolds, M., Bennett, J., and Boyer, J. (2009) Conceptual framework for drought phenotyping during molecular breeding. *Trends Plant Sci.*, **14** (9), 488–496.

176 Finkel, E. (2010) Genetic resources. Parlous times for seed banks spell trouble for Australian agriculture. *Science*, **329** (5999), 1591.

177 Furbank, R.T. (2009) Plant phenomics: from gene to form and function. *Funct. Plant Biol.*, **36** (11), v–vi

178 Nagel, K.A., Kastenholz, B., Jahnke, S., van Dusschoten, D., Aach, T., Muhlich, M., Truhn, D., Scharr, H., Terjung, S., Walter, A., and Schurr, U. (2009) Temperature responses of roots: impact on growth, root system architecture and implications for phenotyping. *Funct. Plant Biol.*, **36** (11), 947–959.

179 Yazdanbakhsh, N. and Fisahn, J. (2009) High throughput phenotyping of root growth dynamics, lateral root formation, root architecture and root hair development enabled by PlaRoM. *Funct. Plant Biol.*, **36** (11), 938–946.

180 Jansen, M., Gilmer, F., Biskup, B., Nagel, K.A., Rascher, U., Fischbach, A., Briem, S., Dreissen, G., Tittmann, S., Braun, S., De Jaeger, I., Metzlaff, M., Schurr, U., Scharr, H., and Walter, A. (2009) Simultaneous phenotyping of leaf growth and chlorophyll fluorescence via GROWSCREEN FLUORO allows detection of stress tolerance in *Arabidopsis thaliana* and other rosette plants. *Funct. Plant Biol.*, **36** (11), 902–914.

181 Moran, M.S., Inoue, Y., and Barnes, E.M. (1997) Opportunities and limitations for image-based remote sensing in precision crop management. *Remote Sens. Environ.*, **61** (3), 319–346.

182 Möller, M., Alchanatis, V., Cohen, Y., Meron, M., Tsipris, J., Naor, A., Ostrovsky, V., Sprintsin, M., and Cohen, S. (2007) Use of thermal and visible imagery for estimating crop water status of irrigated grapevine. *J. Exp. Bot.*, **58** (4), 827–838.

183 Berger, B., Parent, B., and Tester, M. (2010) High-throughput shoot imaging to study drought responses. *J. Exp. Bot.*, **61** (13), 3519–3528.

184 Rajendran, K., Tester, M., and Roy, S.J. (2009) Quantifying the three main components of salinity tolerance in cereals. *Plant Cell Environ.*, **32** (3), 237–249.

185 Sadok, W., Naudin, P., Boussuge, B., Muller, B., Welcker, C., and Tardieu, F. (2007) Leaf growth rate per unit thermal time follows QTL-dependent daily patterns in hundreds of maize lines under naturally fluctuating conditions. *Plant Cell Environ.*, **30** (2), 135–146.

Section IIB Omics

Improving Crop Resistance to Abiotic Stress, First Edition.
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10
Functional Genomics of Drought Tolerance in Crops: Engineering Transcriptional Regulators and Pathways

Bala Rathinasabapathi

Drought stress is a major limitation for crop productivity worldwide, especially in irrigated agriculture. One solution to this problem is the use of cultivars that are tolerant to drought. However, conventional breeding to improve drought tolerance has proven difficult because drought and dehydration tolerance in plants are not fully understood. As a result of research during the past two decades, networks of genes participating in plant adaptations to drought stress have been identified. In this chapter, I present this research progress and indicate the use of specific genes to engineer crops for improved drought tolerance.

10.1
Introduction

Drought stress causes severe devastation to crop productivity worldwide. Economic losses due to the occurrence of a combination of drought and high-temperature stress on crops are enormous. For example, one estimate for economic losses caused by drought and high-temperature stress for a 5-year period (2005–2009) totaled up to US $19 billion in the United States [1].

Because water resources are becoming limited, genetic improvement of crops for increased tolerance to drought has become an important aim in agricultural research. Drought and high-temperature tolerance in plants are complex traits with various interacting components. These include plant adaptations related to cellular tolerance to oxidative, osmotic, and high-temperature stress. Other adaptations relate to physiological features that enhance water uptake by the roots, and adaptations that decrease the loss of water through transpiration. Developmental adaptations related to flowering time and partitioning of biomass between leaves and grain are also important for crop productivity under stress. Some progress has been made by the use of conventional breeding to improve drought tolerance in important crops such as rice [2], corn [3], cowpea [4], and chickpea [5], but the use of recombinant DNA and genomic methods are likely to be crucial in the near future.

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With the availability of genome sequence data for plants, microbes, and other organisms, and technologies to transfer and express foreign genes in crop plants, metabolic engineering has become an important tool for improving crops for tolerance to drought and high-temperature stress. Much of our current understanding of the genes playing roles in drought tolerance has come from the studies of model species such as *Arabidopsis thaliana* and rice. High-throughput functional genomic tools to assess gene and protein expression in response to stress have greatly contributed to rapid advances in this area. The objective of this chapter is to highlight some of the most successful metabolic engineering attempts in improving drought and high-temperature tolerance in crops by using rice as a crop model and suggest future opportunities with the greatest promise.

10.2
Transcriptional Regulation of Stress Signaling Networks

Upon stress, specific networks of plant genes are upregulated leading to improved plant tolerance to stress. In a metabolic engineering strategy termed "regulon engineering," ectopic expression of specific transcription factors (TFs) at the early part of a signaling cascade is used to upregulate the transcription of a number of genes resulting in stress tolerance [6, 7]. An estimated 1300 TFs are known in rice genome and about 45% are from gene families specific to plants [8]. Ray *et al*. [9] showed that in rice 5611 transcripts were modulated by water-deficit stress conditions [8]. Among about 50 families of TFs, several have been identified to have possible roles in drought-stress tolerance. Members of the zinc finger TFs were upregulated under drought and NAC family transcription factors were upregulated by multiple abiotic stresses such as water deficit, salt, and cold stress [9].

However, constitutive expression of the TFs often results in stunted growth and other developmental problems in the plant [10]. Expression of TFs under stress-inducible promoters such as RD29a, OsNAC6, and HSP101 have eliminated or reduced these problems [10]. Many early studies concentrated on evaluating transgenic plants of model species for stress tolerance under laboratory conditions. These have suggested roles for specific transcription factors in drought tolerance. Recent studies, however, have evaluated transgenic crops expressing TFs under the control of inducible or tissue-specific promoters, under field drought conditions [11–13]. Table 10.1 lists examples for successful regulon engineering to improve drought and high-temperature stress tolerance in rice, although much of the basic information on the transcription factors emerged from studies in the model plant *A. thaliana*.

10.2.1
ABA-Inducible Transcription Factors

The growth regulator abscisic acid (ABA) is synthesized from the C40 carotenoid precursor phytoene [20]. The step that leads to zeaxanthin, catalyzed by β-carotene

Table 10.1 Examples of successful regulon engineering to improve drought stress tolerance in cultivated rice.

Gene	Transcriptional factor	Promoter	Parameters evaluated	References
AtABF3	bZIP	ZmUbi1	Leaf rolling, wilting, photosynthesis	[14]
OsWRKY11	WRKY, Zn-finger	HSP101	Leaf wilting, water loss	[15]
OsNAC6	NAC	ZmUbi, OsNAC6, and Lip9	Survival after root-drying treatment, salt tolerance	[10]
OsDREB1A	AP2/ERF	Actin 1, HVA22p	Spikelet fertility, relative yield, photosynthesis	[12, 14, 16]
OsNAC10	NAC	GOS2, RCc3	Relative grain yield	[11, 13]
AtHardy	AP2/ERF	35S	Water use efficiency, transpiration, photosynthesis	[17]
OsZFP252; DST	TFIIIA-type Zn finger	35S, LOF	Proline and sugars, hydrogen peroxide	[18, 19]

hydroxylase (BCH), has been shown to be critical for drought tolerance and oxidative stress resistance [21]. Figure 10.1 shows the enzymes participating in ABA synthesis beginning from zeaxanthin [20]. ABA is catabolized via hydroxylation and conjugation to glucose to produce less active forms (Figure 10.1). Although ABA concentration in plants is regulated at multiple levels, upon drought stress induction of 9-*cis*-epoxycarotenoid dioxygenase especially in the vascular tissue has been observed [22, 23]. This step should be a good target for metabolic engineering.

Transcription factors named "ABA-responsive element binding proteins/factors (ABREB/ABF)" are part of a family of basic leucine zipper (bZIP) TFs. Upon ABA signaling, an activated form of AREB/ABF binds to the conserved regulatory *cis*-element sequence ACGTGT/GC to induce gene expression [24]. Several aspects of ABA-regulated gene networks have been elucidated in the last decade. Figure 10.2 shows the gene products known in the ABA signaling network involved in plant tolerance to drought stress. Poly ADP-ribose polymerase (PARP), an NAD-dependent enzyme known to be induced by drought and ionizing radiation [25], mediates a unique posttranslational protein modification by tagging long-branched poly(ADP-ribose) polymers to nuclear target proteins. Silencing of PARP resulted in a broad-spectrum stress resistance partly due to an increase in ABA levels (Figure 10.2), and this strategy has been used in building drought-tolerant transgenic crops [26].

Type 2C protein phosphatases ABI1 and HAB1 negatively regulate ABA response. There are proteins named "regulatory components of ABA receptors, RCARs" that bind to ABI1 and HAB1 [27] that act as ABA sensors. Farnesyltransferase, ERA1, expressed in the guard cells [28], proteins involved in mRNA splicing, export, and degradation such as mRNA cap binding protein ABH1 [29] and Sm-like SnRNP

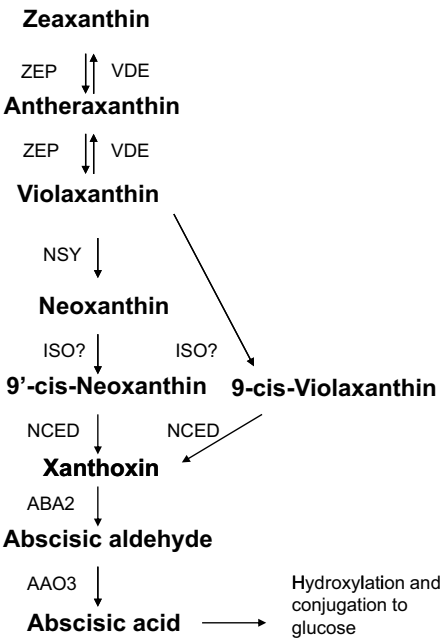

Figure 10.1 Synthesis and catabolism of abscisic acid. The enzymes are ZEP: zeaxanthin epoxidase; VDE: violaxanthin deepoxidase; NSY: neoxanthin synthase; ISO: isomerase; NCED: 9-*cis*-epoxycarotenoid dioxygenase; ABA2: short-chain alcohol dehydrogenase; and AAO3: aldehyde oxidase.

protein, SAD1, are negative regulators of ABA signaling [30]. PYR1 and other ABA receptors PYL1 and PYL2 bind to ABA to inhibit type 2C protein phosphatases, thus controlling SnRK2 kinase activity [30–32]. PYR1's role in negative regulation on ABA signaling was discovered by the use of a synthetic growth inhibitor pyrabactin [33] employing a chemical genetics approach.

Other inhibitory factor DOR, a putative F-box protein interacting with ASK14 and CUL1, is a negative regulator of ABA-mediated stomatal closure [34]. When two negative regulators of ABA signaling were mutated simultaneously [35], the double mutants showed strong hypersensitivity to ABA, and decreased water loss, suggesting this approach to be a way to improve crop performance under drought.

ABA signaling is also regulated via protein modifications and degradations. Isoprenylated proteins contain an isoprenylcysteine methyl ester at the C-terminus. An isoprenylcysteine methyltransferase (ICMT) is a negative regulator of ABA signaling [36]. Recently, an E3 ubiquitin ligase (AIRP1), a cytosolic protein involved in degradation of ubiquitinated proteins, was demonstrated to function as a positive regulator of ABA-dependent response to drought stress [37]. In contrast, E3 ligase KEG (KEEP ON GOING), which ubiquitinates ABI5, negatively regulates ABA signaling [38]. ABA promotes ABI5 accumulation by inducing the ubiquitination and degradation of KEG [39]. A multitude of regulatory controls on ABA-mediated signaling has given a number of new technologies to engineer crops for improved drought tolerance [40].

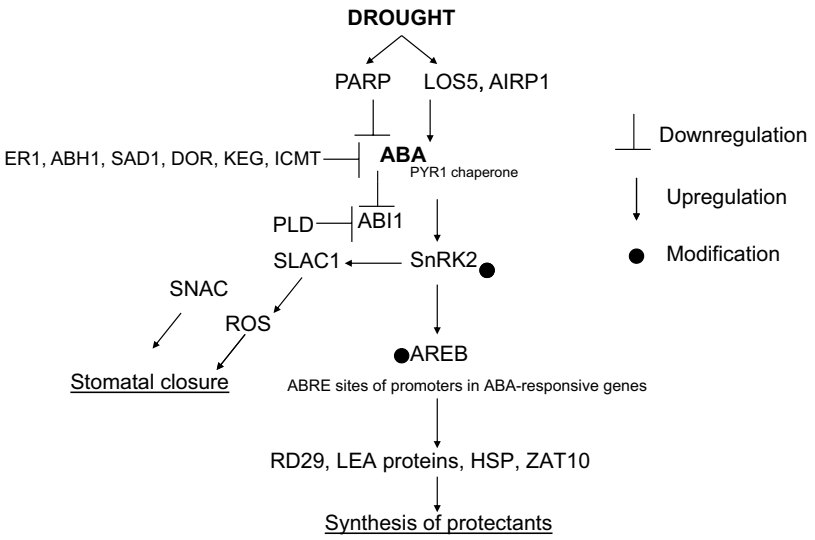

Figure 10.2 ABA-dependent signaling pathway leading to drought tolerance in plants. AIRP1: ABA-insensitive RING protein1; PARP: poly(ADP-ribose) polymerase; LOS5: molybdenum cofactor sulfurase; PYR1: ABA receptor; PLD: phospholipase D; SLAC1: slow anion channel-associated 1; ABI1: type 2C protein phosphatase; ER1: farnesyltransferase β-subunit; ABH1: mRNA cap-binding protein; ICMT: isoprenylcysteine methyltransferase; SAD1: Sm-like SnRNP protein; SnRK2: kinase; AREB: ABA-responsive element binding protein; LEA: dehydrin; HSP: heat shock protein; ZAT10: salt tolerance zinc finger; RD29A: responsive to desiccation 29A.

However, antagonistic crosstalk between ABA signaling and systemic acquired resistance (SAR), mediated by salicylic acid [41], suggests that protecting crops from drought stress damage via modulating ABA-signaling could have unintended consequences on crop tolerance to biotic stress. More research is needed to examine this point.

10.3
ABA-Independent Signaling Networks

10.3.1
NAC Transcription Factors

Plants also regulate stress tolerance-related genes via ABA-independent pathways. Figure 10.3 shows the gene products involved in ABA-independent response to drought stress. NAM, ATAF, and CUC (NAC) transcription factors contain a highly conserved N-terminal DNA binding domain and a variable C-terminal domain [42]. Stress-related NAC TFs (SNACs) belong to group III, phylogenetically distinct from four other groups [43]. Specific NAC TFs induced by stress were identified in different plant species. SNAC1 in *Arabidopsis* is expressed in the stomata. Transgenic crops overexpressing SNAC1 protein had improved tolerance to drought [11].

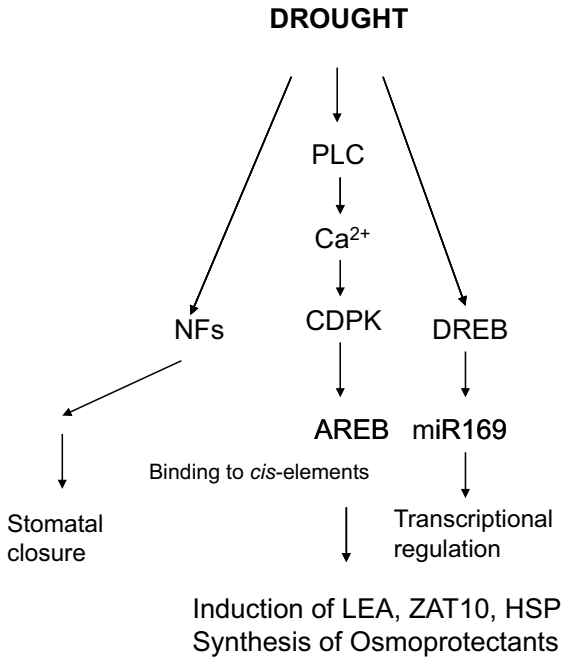

Figure 10.3 ABA-independent signaling pathway for drought stress tolerance. PLC: phospholipase C; CDPK: Ca-dependent protein kinase; AREB: ABA-responsive element binding protein; DREB: dehydration-responsive element binding; SNAC: stress-responsive NAC; miR169: microRNA169; NFs: nuclear factor Y proteins.

10.3.2 DREB/CBF Transcription Factors

Yamaguchi-Shinozaki and Shinozaki identified TFs that are induced by drought but are not under ABA control. Some of these ABA-independent TFs (DREB1/CBF) are involved in cold tolerance and DREB2 (dehydration-responsive element binding 2) is important in drought tolerance [24, 43]. DREB2s interact with a *cis*-acting DRE/CRT (C-repeat) sequence to activate the expression of downstream genes. Overexpression of DREB2-type transcription factors has been used as a strategy to achieve improved tolerance to drought in many crop species [43–46]. In rice, and in other cereals, two types of transcripts of DREB2B – active and inactive – are produced by alternative splicing. Upon stress, the relative amounts of active transcripts increase, likely by a regulation on the splicing machinery [46].

MicroRNAs are small noncoding single-stranded RNAs that regulate gene expression by target mRNA cleavage and translational repression. Zhao *et al.* [47] showed that in rice miR-169g was the only member induced by drought in the miR-169 family [47] and they suggested that miR-169g could transiently inhibit NF-YA transcription factor [48].

Although manipulation of TFs would continue to be an important strategy for achieving drought-tolerant plants, its limitation in its present form would be that only a level of stress tolerance already available within the species could be achieved. While further intricate details on the roles of genes involved in these stress regulatory pathways will continue to emerge, we also need studies searching for structural genes involved in pathways leading to stress tolerance in naturally stress-tolerant species. Future metabolic engineering strategies should combine the manipulation of transcriptional network and introduce novel pathways from stress tolerant species.

10.4
Pathways for Osmoprotectant Synthesis

Many plants synthesize small molecules in response to stress. Osmoprotectants are compatible solutes usually derived from amino acids and sugars. Amino acid proline; the quaternary ammonium compounds glycine betaine, proline betaine, and β-alanine betaine; sugar alcohols mannitol, sorbitol, and trehalose; and raffinose family oligosaccharides have been identified as osmoprotectants in plants [49]. Accumulation of osmoprotectants could have multiple benefits for the plant including osmotic adjustment, turgor maintenance, and protection of proteins, DNA, and membranes from damage by stress [50]. A series of studies on engineering plants for glycine betaine overproduction has identified constraints in the availability of the precursor choline and its import into chloroplasts [51]. Engineering plants for overproduction of glycine betaine has resulted in plants with improved drought tolerance [52, 53]. In another study, simultaneous stress-inducible expression of choline oxidase (for glycine betaine synthesis) and superoxide dismutase and ascorbate peroxidase (to improve oxidative stress tolerance) resulted in transgenic potato plants with increased tolerance to drought [54]. In *Arabidopsis*, raffinose family oligosaccharides galactinol and raffinose may function as osmoprotectants against drought stress [55] and oxidative damage [56]. Transgenic plants overexpressing DREB genes had increased levels of proline suggesting that proline synthesis may be under the control of DREB. A comparative functional genomics study on maize genotypes differing in drought tolerance concluded that sugar synthesis, sugar transport, and cell injury prevention are key factors in determining drought tolerance [57]. Studies are needed to link the signaling pathways for stress to understand how osmoprotectant synthesis, accumulation, and degradation are regulated both at the transcriptional and at the translational levels.

10.5
Transporters

Drought stress tolerance can be improved if solute concentration in the vacuole could be increased, thus favoring water uptake. Apse *et al.* [58] reported a strategy to increase

the activity of a vacuolar sodium/proton antiporter, thus improving plant tolerance to salinity stress [58]. Similarly, when the activity of H + pump on the vacuolar membrane (vacuolar H + pyrophosphatase) is increased, the plants gain improved tolerance to both salinity and drought [59]. This strategy has now been applied to improve drought tolerance in a number of crops including cotton [60, 61] and maize [62].

Aquaporins (or major intrinsic proteins, MIPs) facilitate water transport over cellular membranes. In plants, they occur as a family of 30–35 proteins classified as tonoplast intrinsic proteins (TIPs), plasma membrane intrinsic proteins (PIPs), NOD26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs). In *A. thaliana*, many of them were downregulated by drought stress at the transcriptional level, but two PIPs whose promoters had drought stress response elements were upregulated [63], suggesting these two PIPs with possible roles in drought tolerance. Constitutive overexpression of PIPs in transgenic plants improved vigor under favorable growth conditions, but not under drought or salt stress [64]. More research is needed to engineer stress-regulated PIPs.

10.6
Combination of Drought and High-Temperature Stress and Oxidative Stress

In the field, drought often occurs together with high-temperature stress. The biology of the combination of these two stress factors on plants has not been examined at the molecular level, although it is well known that both drought and high-temperature stress induce oxidative damage. Ectopic expression of single transgenes involved in osmoprotection or oxidative stress tolerance has resulted in plants with improved tolerance to high-temperature stress [65, 66]. Plant breeders have recognized that genotypes that are resistant to oxidative stress are also resistant to drought and high-temperature stress [67], providing a correlating evidence for the importance of oxidative stress tolerance. Functional genomic studies on naturally dehydration tolerant species and the effects of simultaneous application of drought and high temperature on plants have revealed key roles for antioxidant enzymes in both drought and high-temperature stress tolerance [68, 69]. Future work is needed to identify differences, similarities, and overlaps between signaling pathways leading to high-temperature stress and those leading to drought tolerance.

10.7
Conclusions

Although *Arabidopsis* and model crop rice have contributed to our understanding of how plants adapt to drought and high-temperature stress, time is ripe for beginning functional genomic studies on species that are naturally tolerant to stress. Wild relatives of major crops could be a starting point [70], although they have not been used extensively in conventional breeding. Drought tolerance has been identified in wild emmer (*Triticum dicoccoides*) [71], certain barley genotypes from Tibet [72], wild

Oryza spp. and accessions of cultivated rice [73], and pearl millet (*Pennisetum glaucum*) [74]. Analysis of orthologues of *Arabidopsis* and rice genes with key roles in drought stress tolerance pathways in these wild grasses should be useful both to uncover novelties and to improve cereal crops for drought tolerance.

Understanding drought tolerance in plants is a long-term goal as this is a valuable but complex trait. Genome sequences, high-throughput technologies such as microarrays, metabolomics, and proteomics have provided us unprecedented tools with capabilities to identify genes and gene products with potential roles in drought tolerance. However, functional tests on specific genes using mutant and overexpression lines are still laborious and time consuming. To facilitate these tests, new rapid technologies to create and analyze stable transgenic lines in major crops are needed. Despite that, as illustrated above, transgenic crops improved for drought tolerance with great promise are tested under field conditions and perhaps represent the first steps toward our next green revolution.

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References

1 NESDIS (2010) Billion Dollar US Weather Disasters. NOAA Satellite and Information Service, US Department of Commerce. Available at http://www.ncdc.noaa.gov/oa/reports/billionz.html#chron. (Accessed 15 Feb 2011).

2 Serraj, R., Kumar, A., McNally, K.L. *et al.* (2009) *Adv. Agron.*, **103**, 41–98.

3 Bruce, W.B., Edmeades, G.O., and Barker, T.C. (2002) *J. Exp. Bot.*, **53**, 13–25.

4 Agbicodo, E.M., Fatokun, C.A., Murannaka, S. *et al.* (2009) *Euphytica*, **167**, 353–370.

5 Singh, K.B., Malhotra, R.S., Haliala, M.H. *et al.* (1994) *Euphytica*, **73**, 137–149.

6 Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G. *et al.* (1998) *Science*, **280**, 104–106.

7 Kasuga, M., Liu, Q., Miura, S. *et al.* (1999) *Nat. Biotechnol.*, **17**, 287–291.

8 Kikuchi, S., Satoh, K., Nagata, T. *et al.* (2003) *Science*, **301**, 376–379.

9 Ray, S., Dansana, P.K., Giri, J. *et al.* (2010) *Funct. Integr. Genomics*, **11**, 157–178.

10 Nakashima, K., Tran, L.S., Van Nguyen, D. *et al.* (2007) *Plant J.*, **51**, 617–630.

11 Hu, H., Dai, M., Yao, J. *et al.* (2006) *Proc. Natl. Acad. Sci. USA*, **35**, 12987–12992.

12 Xiao, B.Z., Chen, X., Xiang, C.B. *et al.* (2009) *Mol. Plant*, **2**, 73–83.

13 Jeong, J.S., Kim, S.Y., Baek, K.H. *et al.* (2010) *Plant Physiol.*, **153**, 185–197.

14 Oh, S.J., Song, S.I., Kim, Y.S. *et al.* (2005) *Plant Physiol.*, **138**, 341–351.

15 Wu, X., Shiroto, Y., Kishitani, S. *et al.* (2009) *Plant Cell Rep.*, **28**, 21–30.

16 Wang, Q., Guan, Y., Wu, Y. *et al.* (2008) *Plant Mol. Biol.*, **67**, 589–602.

17 Karaba, A., Dixit, S., Greco, R. *et al.* (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 15270–15275.

18 Xu, D.Q., Huang, J., Guo, S.Q. *et al.* (2008) *FEBS Lett.*, **582**, 1037–1043.

19 Huang, X.Y., Chao, D.Y., Gao, J.P. *et al.* (2009) *Genes Dev.*, **23**, 1805–1817.

20 Nambara, E. and Marion-Poll, A. (2005) *Plant Biol.*, **56**, 165–185.

21 Du, H., Wang, N., Cui, F. *et al.* (2010) *Plant Physiol.*, **154**, 1304–1318.

22 Iuchi, S., Kobayashi, M., Taji, T. *et al.* (2001) *Plant J.*, **27**, 325–333.

23 Endo, A., Sawada, Y., Takahashi, H. *et al.* (2008) *Plant Physiol.*, **147**, 1984–1993.
24 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) *Plant Biol.*, **57**, 781–803.
25 Doucet-Chabeaud, G., Godon, C., Brutesco, C. *et al.* (2001) *Mol. Genet. Genomics*, **265**, 954–963.
26 Vanderauwera, S., Block, D.M., and Steene, N.V. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 15150–15155.
27 Ma, Y., Szostkiewicz, I., Korte, A. *et al.* (2009) *Science*, **324**, 1064–1068.
28 Pei, Z.M., Ghassemian, M., Kwak, C.M. *et al.* (1998) *Science*, **282**, 287–290.
29 Hugouvieux, V., Murata, Y., Young, J.J. *et al.* (2002) *Plant Physiol.*, **130**, 1276–1287.
30 Xiong, L., Gong, Z., Rock, C.D. *et al.* (2001) *Dev. Cell*, **1**, 771–781.
31 Santiago, J., Dupeux, F., Round, A. *et al.* (2009) *Nature*, **462**, 665–669.
32 Weiner, J.J., Peterson, F.C., Volkman, B.F. *et al.* (2010) *Curr. Opin. Plant Biol.*, **13**, 495–502.
33 Park, S.Y., Fung, P., Nishimura, N. *et al.* (2009) *Science*, **324**, 1068–1071.
34 Zhang, Y., Xu, W., Li, Z. *et al.* (2008) *Plant Physiol.*, **148**, 2121–2133.
35 Saez, A., Robert, N., Maktabi, M.H. *et al.* (2006) *Plant Physiol.*, **141**, 1389–1399.
36 Huizinga, D.H., Omosegbon, O., Omery, B. *et al.* (2008) *Plant Cell*, **20**, 2714–2728.
37 Ryu, M.Y., Cho, S.K., and Kim, W.T. (2010) *Plant Physiol.*, **154**, 1983–1997.
38 Liu, H. and Stone, S.L. (2010) *Plant Cell*, **22**, 2630–2641.
39 Somvanshi, V.S. (2009) *Recent Pat. DNA Gene Seq.*, **3**, 16–25.
40 Yasuda, M., Ishikawa, A., Jikumaru, Y. *et al.* (2008) *Plant Cell*, **20**, 1678–1692.
41 Olsen, A.N., Ernst, H.A., Leggio, L.L. *et al.* (2005) *Trends Plant Sci.*, **10**, 79–87.
42 Fang, Y., You, J., Xie, K. *et al.* (2008) *Mol. Genet. Genomics*, **280**, 547–563.
43 Sakuma, Y., Maruyama, K., Qin, F. *et al.* (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 18822–18827.
44 Morran, S., Eini, O., Pyvovarenko, T. *et al.* (2011) *Plant Biotechnol. J.*, **9**, 230–249.
45 Chen, J.Q., Meng, X.P., Zhang, Y. *et al.* (2008) *Biotechnol. Lett.*, **30**, 191–198.
46 Matsukura, S., Mizoi, J., Yoshida, T. *et al.* (2010) *Mol. Genet. Genomics*, **283**, 185–196.
47 Zhao, B., Liang, R., Ge, L. *et al.* (2007) *Biochem. Biophys. Res. Commun.*, **354**, 585–590.
48 Zhao, B., Ge, L., Liang, R. *et al.* (2009) *BMC Mol. Biol.*, **10**, 29–39.
49 Rathinasabapathi, B. and Kaur, R. (2006) Metabolic engineering for stress tolerance, in *Physiology and Molecular Biology of Stress Tolerance* (eds K.V.M. Rao, A.S. Raghavendra, and J.K. Reddy), Springer, The Netherlands, pp. 255–299.
50 Rontein, D., Basset, G., and Hanson, A.D. (2002) *Metab. Eng.*, **4**, 49–56.
51 Nuccio, M.L., McNeil, S.D., Ziemak, M.J. *et al.* (2000) *Metab. Eng.*, **2**, 300–311.
52 Quan, R., Shang, M., Zhang, H. *et al.* (2004) *Plant Biotechnol. J.*, **2**, 477–486.
53 Shen, B., Hohmann, S., Jensen, R.G., and Bohnert, H.J. (1999) *Plant Physiol.*, **121**, 45–52.
54 Ahmad, R., Kim, Y.H., Kim, M.D. *et al.* (2010) *Physiol. Plant.*, **138**, 520–533.
55 Taji, T., Ohsumi, C., Iuchi, S. *et al.* (2002) *Plant J.*, **29**, 417–426.
56 Nishizawa, A., Yabuta, Y., and Shigeoka, S. (2008) *Plant Physiol.*, **147**, 1251–1263.
57 Li, Y., Sun, C., Huang, Z. *et al.* (2009) *Plant Cell Physiol.*, **50**, 2092–2111.
58 Apse, M.P., Aharon, G.S., Snedden, W.A. *et al.* (1999) *Science*, **285**, 1256–1258.
59 Gaxiola, R.A., Li, J., Undurraga, S. *et al.* (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 11444–11449.
60 Lv, S., Zhang, K., Gao, Q. *et al.* (2008) *Plant Cell Physiol.*, **49**, 1150–1164.
61 Pasapula, V., Shen, G., Kuppu, S. *et al.* (2011) *Plant Biotechnol.*, **9**, 88–99.
62 Li, B., Wei, A., Song, C. *et al.* (2008) *Plant Biotech. J.*, **6**, 146–159.
63 Alexandersson, E., Danielson, J.A.H., Rade, J. *et al.* (2010) *Plant J.*, **61**, 650–660.
64 Aharon, R., Shahak, Y., Wininger, S. *et al.* (2003) *Plant Cell*, **15**, 439–447.
65 Fouad, W.M. and Rathinasabapathi, B. (2006) *Plant Mol. Biol.*, **60**, 495–505.
66 Sundaram, S. and Rathinasabapathi, B. (2010) *Planta*, **231**, 361–369.
67 Altinkut, A., Kazan, K., Ipekci, Z., and Gozukirmizi, N. (2001) *Euphytica*, **121**, 81–86.
68 Oliver, M.J., Dowd, S.E., Zargoza, J. *et al.* (2004) *BMC Genomics*, **5**, 89.
69 Rizhsky, L., Daveletova, S., Liang, H. *et al.* (2004) *Plant Physiol.*, **134**, 1683–1696.

70 Hahhar, H. and Hodgkin, T. (2007) *Euphytica*, **156**, 1–13.
71 Xie, W. and Nevo, E. (2008) *Euphytica*, **164**, 603–614.
72 Zhao, J., Sun, H., Dai, H. *et al*. (2010) *Euphytica*, **172**, 395–403.
73 Liu, L., Lafitte, R., and Guan, D. (2004) *Euphytica*, **138**, 149–161.
74 Yadav, R.S., Sehgal, D., and Vadez, V. (2010) *J. Exp. Bot.*, **62**, 397–408.

11
Transcriptomic and Metabolomic Approaches to the Analysis of Plant Freezing Tolerance and Cold Acclimation

Dirk K. Hincha, Carmen Espinoza, and Ellen Zuther

Freezing tolerance is an important trait in plants because it limits the geographical distribution of wild species and the growth performance and yield of crop plants. Plants from temperate climates are able to increase their freezing tolerance by a process called cold acclimation. This process is driven by a complex reprogramming of the plant transcriptome and metabolome. In the last decade, several transcript and metabolite profiling studies on the model species *Arabidopsis thaliana* and on crop plants and trees have been reported. These studies point not only to many molecular changes during cold acclimation that are common to most or all investigated species but also to more species-specific changes. In addition, some profiling studies allow a first estimate of the relative importance of transcriptional regulation compared to translational and posttranslational regulation of plant metabolism in the cold.

11.1
Introduction

Cold has a major influence on plant growth and survival, limiting the geographical distribution of natural species and the yield and growing season of agricultural crops. Considerable effort has, therefore, been directed toward understanding how plants respond and adapt to low temperature. While plants of tropical or subtropical origin are sensitive to low temperatures above the freezing point (chilling sensitive), plants adapted to cooler climates are chilling resistant. Even at temperatures below 0 °C, they show damage only when their tissues freeze, that is, after ice crystallization has occurred. Freezing damage is therefore in general not a consequence of low temperatures *per se* but rather the result of cellular dehydration brought about by extracellular ice crystallization (see Refs [1–3] for reviews). The major sites of freezing injury in plants are the cellular membranes. There is extensive evidence for damage to the plasma membrane [1, 4], to chloroplast thylakoid, and to envelope membranes during a freeze–thaw cycle [5–7]. However, it can be expected that other cellular membrane systems are also damaged by freezing, and inactivation of sensitive enzymes cannot be excluded, although evidence to this effect is still lacking.

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Freezing tolerance can differ strongly depending on the thermal history of the plants. Nonacclimated tolerance denotes the capacity to survive a freezing event without prior adaptation. Acclimated tolerance is usually higher and reflects the ability of many plant species from temperate climates to increase their freezing tolerance in response to low but nonfreezing temperatures. Subzero acclimation can lead to an additional increase in freezing tolerance after cold acclimation when plants are exposed to a mild, nondamaging frost. The molecular basis of nonacclimated tolerance is poorly understood although it has been reported that it may be genetically determined by loci independent of acclimated tolerance in potato [8], willow [9], and oilseed rape [10, 11]. Equally, while subzero acclimation has been repeatedly described, mainly in cereals [12–16], and also in the model plant *Arabidopsis thaliana* [17, 18], no large-scale profiling or genetic mapping studies have been published in this area and no key genes or metabolites have yet been identified.

Using the barley Affymetrix microarray [19], the gene expression of wheat was compared under nonacclimated, cold acclimated, and subzero acclimated conditions [12]. Up to now this is the only transcriptomic study of subzero acclimation, which, however, suffers from the restrictions inherent to cross species microarray hybridization, in particular with arrays that use short oligonucleotides for hybridization. Nevertheless, this study did clearly show that the additional freezing tolerance obtained during subzero acclimation is the result of complex biological processes that, at least at the level of gene expression, are clearly different from cold acclimation. In the future, more comprehensive studies will be necessary to draw firm functional conclusions for the understanding of subzero acclimation.

In contrast, cold acclimation is well studied in a large range of both wild and crop plant species and involves a wide array of metabolic changes governed by extensive reprogramming at the level of gene expression. Consequently, this chapter will focus on transcriptomic and metabolomic studies of cold acclimation and the associated increase in freezing tolerance.

The degree of freezing tolerance differs vastly between plant species, from around $-1\,^{\circ}C$ to $-2\,^{\circ}C$ in some tender plants such as tobacco [20] to the temperature of liquid nitrogen ($-196\,^{\circ}C$) in some hardy trees and shrubs [21–23]. In addition, most plants from temperate climates follow an annual cycle of acclimation and deacclimation, with their maximum freezing tolerance in winter and the minimum during summer.

In herbaceous plants, acclimation is triggered by exposure to low, nonfreezing temperatures, usually in the range between 10 and $0\,^{\circ}C$ over several days and increases the freezing tolerance in different species by approximately 2–25 °C [4, 22, 24–27]. In woody species, photoperiod is an additional determinant of acclimation, with a combination of low temperature and short days inducing the highest freezing tolerance [28, 29]. The freezing tolerance of cold acclimated plants is a multigenic and quantitative trait. From QTL mapping studies in various species [9–11, 30–37], it is obvious that many genetic loci contribute to the freezing tolerance of any given species and that the loci differ even between different mapping populations within a species. In addition, it has recently been shown that there is large variation in the freezing tolerance of different geographical populations (accessions) within a single species (*A. thaliana*; [25, 38–40]). Such natural

genetic variation, which is also present, for example, in breeding lines and cultivars of crop species, is an excellent basis to study the molecular mechanisms underlying complex quantitative traits [41, 42].

The genetic complexity of plant freezing tolerance is reflected in the complex physiological and biochemical changes associated with cold acclimation in plants. These changes are extensive and include growth and water balance, the accumulation of compatible solutes, changes in membrane and cell wall composition, increased antioxidant production, and changes in gene expression and protein levels (see Refs [1–3, 43] for reviews).

Especially low-temperature signal transduction and the resulting regulation of cold-responsive gene expression have received much attention in recent years. The main breakthroughs in this area have come from molecular genetic studies in the model plant species *A. thaliana* and this field has been reviewed repeatedly (e.g., [44–48]). Forward and reverse genetics have defined some of the key regulatory components of cold acclimation in *Arabidopsis*. A prominent role has been demonstrated for the *C-repeat binding factors (CBF) 1*, *2*, and *3* [49], which are also known as *dehydration-responsive element binding1 (DREB1)b*, *c*, and *a* (At4g25490, At4g25470, At4g25480), respectively [50]. These AP2/ERF type transcription factors are rapidly induced in response to cold and reach a peak of expression after 2–3 h of exposure to cold [38, 49–51]. The CBF proteins, in turn, activate the expression of a set of target genes by binding to a core sequence in their promoters, called the C-repeat (CRT), the dehydration-responsive element (DRE), or the low-temperature response element (LTRE), which is involved in cold responsiveness [52, 53]. Genes containing this motif have been denoted as *COR* (cold-regulated), *(E)RD* ((early) responsive to dehydration), *KIN* (cold-induced), or *LTI* (low-temperature-induced) genes, and are collectively referred to as the "CBF regulon." It has been estimated that 12–20% of all cold-induced transcriptional changes in *Arabidopsis* are accounted for by the action of CBF1–3 [54]. Moreover, it has been found that overexpression of any individual *Arabidopsis CBF* gene leads to constitutive expression of CBF regulon genes and an increase in freezing tolerance without cold exposure [51, 55, 56].

The overexpression of *CBF* genes induces many cold-regulated downstream genes and increases the contents of metabolites under nonacclimating conditions that also accumulate in response to cold [55, 57–59]. The overexpression studies also indicated that the activities of the three transcription factors might be functionally redundant [55]. There is, however, evidence for negative regulation among the *CBF* genes. Analysis of a *CBF2* null mutant in the *Arabidopsis* accession Col-0 indicated that CBF2 might negatively regulate *CBF1* and *CBF3* expression [60, 61]. A recent analysis of the expression of the *CBF* genes in RNAi lines, generated to downregulate the expression of all three genes in different accessions, however, indicated that this regulatory function of CBF2 was evident in Col-0, but not in several other accessions [34], indicating that mechanisms of gene regulation may differ between accessions of the same species.

Reduced *CBF3* expression in the *ice1* mutant was associated with enhanced *CBF2* expression after 6–12 h of cold treatment [62], pointing to a possible compensatory

effect. *Inducer of CBF expression 1* (*ICE1*) encodes a MYC-like bHLH transcriptional activator that is not cold induced at the transcript level and acts upstream of the *CBF* genes enhancing specifically *CBF3* expression in the cold [62]. In addition, ICE1 also seems to be involved in stomatal differentiation [63] through a presumably unrelated signal transduction pathway. In the *ice1* mutant, several genes in the CBF regulon are no longer cold induced [64]. *High expression of osmotically responsive genes 1* (*HOS1*), encoding a RING finger motif protein, negatively regulates the *CBF* genes [65] by mediating ubiquitination and degradation of ICE1 [66], while the presence of the SFR6 (sensitive to freezing 6) protein, which is itself also not strongly cold induced, is required for the induction of downstream genes through an as yet unknown mechanism [67]. CBF-dependent roles in freezing tolerance have also been demonstrated for *low expression of osmotically responsive genes 1* (*LOS1*), a translational elongation factor 2 gene, whose product is involved in protein synthesis in the cold [68] and for *LOS4*, a gene encoding a DEAD box RNA helicase that is essential for mRNA export [69]. In addition, *ZAT12* was shown to downregulate the expression of the *CBF* genes and to have a cold-responsive regulon that partially overlaps with that of CBF2 [70]. While the CBF-related signal transduction pathway is by now fairly well characterized, it should not be forgotten that it does not regulate more than approximately 20% of all cold-regulated genes [54]. It has, for instance, been suggested that the gibberellin-regulated DELLA proteins [71] not only function in reducing plant growth in the cold but also directly contribute to cold acclimation through a mechanism that does not involve the CBF regulon [72]. Other regulators that function independent of the CBFs, such as ESK1 (eskimo1) [73], are not as much studied. Clearly, the transcriptional regulation of cold-responsive gene expression is complex and still only partly understood.

CBF genes, however, appear to be ubiquitous in plant species and are almost always present as gene families. In *Arabidopsis*, there are four characterized *CBF* genes, *CBF1*, *2*, and *3*, located in a tandem array on chromosome 4, are cold induced, while *CBF4* is reported to be involved in drought tolerance [74]. Homologues have been described in many species including wheat, rye, and *Brassica napus*, all of which can acclimate, and even in tomato, which is chilling sensitive [75, 76]. Some species have large *CBF* gene families, for example, barley, which has at least 20 family members [77] of which, however, only a part is cold induced [78]. *CBF* homologues are also present in tree species including poplar [79] and Eucalyptus [80].

During cold acclimation, not only is the plant transcriptome massively remodeled but also are many of these changes in gene expression reflected in biochemical and physiological changes that include increases in the cellular concentration of many metabolites such as sugars and amino acids, which are thought to contribute to the increased freezing tolerance. Such metabolites are generally referred to as osmolytes or compatible solutes.

Compatible solutes are synthesized by many organisms ranging from bacteria to animals and plants, in response to desiccation, osmotic stress, salt stress, or low temperature. This chemically heterogeneous group of substances comprises some amino acids (e.g., proline), quaternary ammonium compounds (e.g., glycine-betaine),

many sugars, sugar alcohols, and several others (see Refs [81, 82] for reviews). Physiologically compatible solutes should have no adverse metabolic effects even at very high concentrations. They are thought not only to stabilize sensitive cellular components such as enzymes and membranes under stress conditions but also to act as bulk osmoprotectants. Therefore, they may act colligatively by increasing the osmotic potential and thereby improving the water status and increasing the cell volume in the frozen state. In addition, they can stabilize macromolecular structures such as proteins by preferential exclusion from the hydration shell of proteins [83], assist refolding of unfolded polypeptides by chaperone proteins [84], and stabilize membranes during freezing and drying [85, 86]. Of particular interest in the context of plant freezing tolerance are raffinose family oligosaccharides [87], fructans [88], proline [89], and glycine-betaine [90, 91]. For example, proline, one of the most studied amino acids in cold acclimation, is able to protect enzymes and other proteins from denaturation during freezing [92, 93]. Photosynthesis can also be affected by oxidative stress-induced damage of photosystems [94]. Sugars can act as ROS scavengers. For instance, ROS-dependent photoinhibition of PSII can be reduced by the accumulation of galactinol and raffinose in transgenic plants [95].

The biochemical, physiological, and genetic studies that have been briefly reviewed above have tremendously advanced our understanding of freezing tolerance and cold acclimation. But since they are usually targeted at specific groups of substances or particular biosynthetic or signal transduction pathways, they will not reveal the whole gamut of the regulatory and metabolic networks that respond to low temperature. The coordinate responses of these networks, however, will ultimately determine the level of freezing tolerance attained by a specific genotype and therefore, for example, the winter survival of a particular cultivar of a crop plant species. The substantial technical advances in the fields of transcript and metabolite profiling technologies over the years have enabled researchers to study plant–environment interactions and adaptation with a much wider scope. High-quality microarrays that cover (almost) all genes in a genome are not only available for model plants such as *A. thaliana* and in addition the recent development of next-generation sequencing methods [96] now enables full transcriptome analysis even for species with very little or no genome sequence information, such as resurrection plants [97].

It is implicit in the interpretation of most transcript profiling studies that the amount of a specific mRNA is quantitatively related to the amount of the encoded protein, its biochemical activity, and its effect on the physiological phenotype. This, however, is an oversimplification and a complete description of the environmental adaptation of a plant such as cold acclimation will require not only an analysis of the abundance of transcripts and metabolites but also information on translation, protein abundance, posttranslational modifications of proteins, protein–protein interactions, and enzyme kinetics, preferably at tissue or cell-type resolution. However, only transcript abundance and the content of central metabolites have been analyzed in sufficient detail until now in profiling studies to draw more general conclusions. Proteomic studies of cold acclimation have been reported less frequently and the number of identified proteins is still comparatively low [98–104]. Only a single

profiling study has so far suggested a link between secondary metabolism and plant freezing tolerance [105].

11.2
Transcriptomic Studies of Plant Cold Acclimation

During the last decade, the analysis of low-temperature-driven changes in the transcriptome of *Arabidopsis* has come into the focus of many research groups, especially after the first description of the CBF signal transduction pathway and the CBF regulon [58]. Around 200 cold-responsive genes could be identified by traditional approaches such as the differential screening of cDNA libraries and Northern blotting (see Ref. [54] for a compilation), but since expression profiling technologies became available, this list has dramatically expanded [25, 54, 106–110]. Furthermore, gene expression profiling technologies have been used to characterize regulatory pathways, including the CBF signal transduction pathway [62, 64, 70, 111]. In some studies, the effects of low temperature were compared with the effects of other abiotic stresses [107, 109, 112], indicating overlapping transcriptional responses to drought, salinity, cold, and abscisic acid (ABA). In addition, gene expression profiling was also combined with metabolite and enzyme activity profiling [25, 113–115] to gain more information on downstream effects of changes in gene expression on metabolic regulation.

Also, the transcriptomic cold responses of crop plants such as wheat [12, 77, 116–118], barley [119, 120], and blueberry [121], as well as those of trees such as poplar [122], Scots pine [123], and Sitka spruce [124] have been investigated during the last years. Most microarray data sets are publicly available, for example, at http://affymetrix.arabidopsis.info/, http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp, and http://www.ncbi.nlm.nih.gov/geo.

11.2.1
Cold-Responsive Genes Identify Cold-Regulated Pathways in Arabidopsis

In the first microarray study of *Arabidopsis* cold acclimation (4 °C for up to 7 days), 306 cold-responsive genes were identified, 218 up- and 88 downregulated, using an Affymetrix array that represented approximately 8000 genes, or less than one-third of the genome [58]. By extrapolation, it was estimated that around 4% of the genome was responsive to low temperature and this estimate was confirmed by a subsequent study [64]. Five hundred and fourteen genes (302 up- and 212 downregulated) were considered as a cold-responsive core set using the Affymetrix ATH1 array containing probe sets for approximately 24 000 *Arabidopsis* genes [70]. Different growth conditions, experimental treatments, and criteria for determining differential gene expression (i.e., different fold-change cutoff values and/or *p*-values from statistical tests with or without multiple testing corrections) resulted in different numbers and identities of cold-responsive genes identified in various

microarray experiments. A comprehensive statistical analysis of gene expression data available at that time [54] indicated that 45% of all transcripts, including genes with small but statistically significant changes in expression, respond to low temperature, in a marked contrast to the previously published estimates of 4 [58] or 14% [107].

When the data were separated into short-, medium-, and long-term responses to low temperature, 808, 1224, and 672 genes were found to be upregulated and 240, 1364, and 915 downregulated in different response categories [54]. In general, differentially expressed genes were divided into two groups, either encoding proteins that presumably directly protect plant cells from freezing damage or that are involved in signal transduction and the regulation of gene expression [125, 126]. Late-embryogenesis abundant (LEA) proteins, enzymes catalyzing the biosynthesis of compatible solutes, antifreeze proteins, chaperones, proteases, enzymes for detoxification of reactive oxygen species (ROS), and lipid desaturases belong to the first group, whereas the second group includes transcription factors, protein kinases, and phosphatases; phospholipases and enzymes involved in phosphoinositide metabolisms; and enzymes involved in the synthesis and degradation of plant hormones [109, 111, 125, 127].

Upregulated genes after exposure to low temperature include the *CBF* transcription factor family and the genes belonging to the CBF regulon [58]. Meta-analysis of available microarray data sets revealed that approximately 15% of all cold-regulated genes and 23% (478 genes) of the upregulated genes are members of the CBF regulon [54], significantly extending earlier estimates of 12% [58] or 38 genes [111]. In the CBF regulon are 41 known or putative transcription factors, mainly from the AP2/EREBP, CO-like, and C2H2 families [54], indicating the complexity of the regulatory pathways downstream of the CBFs.

Approximately, 80% of the cold-responsive genes are not under the control of the CBF transcription factors, indicating the importance of additional regulatory pathways during cold acclimation [54, 128]. Long-term upregulated genes include several known or putative transcription factors (*ZAT12*, *RAV1*, *AtMYB73*, *ATHB-12*, H-protein binding factor 2a, *RAP2.1*, a zinc finger protein, and *RAP2.7*, *ZAT10*, *COL1*, and members of the NAC and basic leucine zipper (bZIP) families) [54, 58, 64, 128]. ZAT12 is a negative regulator of *CBF* expression and its target genes partially overlap with the CBF2 regulon [70]. These transcriptional regulators that are persistently induced under low-temperature conditions might be responsible for maintaining the cold acclimated status. Several genes encoding transcription factors are downregulated as a short-term response to low temperature [58]. However, the fraction of downregulated transcription factor-encoding genes compared to the total number of downregulated genes was much smaller than the corresponding fraction among upregulated genes [64]. Transcription factors of the Aux/IAA and the bHLH families were overrepresented among long-term downregulated genes [54].

Genes encoding protein kinases and phosphatases represented a large group among cold-regulated genes, and in addition the identification of eight upregulated and one downregulated gene encoding enzymes involved in phospholipid signaling

underscores the importance of phospholipid second messengers in cold signaling [64].

At least 55 genes encoding proteins with known or proposed protective functions are cold induced [2, 54]. Many of these proteins can be classified as LEA proteins [129] or hydrophilins [130], showing high hydrophilicity and, in the case of the hydrophilins, a high glycine content.

Another well-known response to low temperature is the accumulation of compatible solutes, such as sugars and proline [131]. Upregulated genes, for example, *P5CS2* and *AtGolS3*, encode parts of the proline and raffinose biosynthesis pathways. Proline synthesis was found to be induced at temperatures below 14 °C, and only a small decrease in temperature was sufficient to induce genes encoding raffinose biosynthesis enzymes [114]. Transcript levels of genes encoding galactinol synthase and raffinose synthase also increase during cold acclimation [113]. During a mild temperature reduction, genes encoding enzymes for sucrose synthesis are cold induced, while genes responsible for sucrose and starch breakdown are repressed [114]. Under short-term cold acclimation conditions, on the other hand, genes encoding enzymes for sucrose and starch degradation (e.g., β-amylase) are upregulated, while in the medium to long term, sucrose degradation by invertase and starch degradation are downregulated [54]. In addition, only one sucrose synthase gene showed a transient increase in expression, but sucrose phosphate synthase transcript levels were generally increased, while transcript levels for sucrose phosphate phosphatase were unchanged [113]. A general increase in the expression of genes encoding enzymes of the TCA cycle was also reported during cold acclimation [54].

After a short-term cold stress, a significant upregulation of genes encoding transport proteins, including ATP binding cassette, sugar and phosphate transporters, and ATPases was found [54, 58]. After a long-term rearrangement of carbohydrate metabolism, sugar transport seems to become less important as indicated by downregulation of genes encoding sugar transporters [54].

The production of reactive oxygen species is induced under cold conditions and the induction of genes encoding ROS scavenging proteins was described after exposure of *Arabidopsis* to low temperature [110]. Likewise, nuclear genes encoding the thylakoid membrane localized early light-inducible proteins (ELIPs) are among the most highly induced genes during cold acclimation [58, 107]. They are thought to function as protectors against photooxidative damage. Enhanced freezing tolerance upon cold acclimation was also connected with the concerted induction of the genes encoding enzymes involved in flavonoid metabolism [25], including the transcription factors PAP1 and PAP2 that are known to regulate this biosynthetic pathway. Flavonoids are also well-known antioxidants [132, 133].

In the cold, growth is reduced and central metabolism is adjusted accordingly. This reduction in growth is an active process that involves the downregulation of photosynthesis-related genes and genes encoding proteins involved in transcription, signaling, and cell wall biogenesis [109]. Downregulation of photosynthesis-related genes is not a main factor in the early response to cold [64] but becomes more important as a medium and long-term adaptation [54], involving also aspects

such as tetrapyrrole synthesis and light signaling. Changes in the expression of photosynthesis-related genes might also reflect adaptations to the low light conditions used in most cold acclimation experiments to prevent photoinhibition [54]. However, a correlation with freezing tolerance also suggests more direct adaptive benefits [25].

Light is a prerequisite of increased freezing tolerance during cold acclimation [134] and it increases the amount of transcripts of cold-responsive genes. The combination of light and cold induces specific genes important for the development of freezing tolerance [110]. A cold treatment in the light induced twice as many genes, including several transcription factors, than a cold treatment in the dark. Two genes encoding AP2 domain transcription factors and novel zinc finger, MYB and NAC transcription factors, are significantly upregulated under cold/light conditions compared to a cold/dark treatment. Cold/light treatment also causes the specific induction of ABA biosynthesis genes, genes encoding enzymes necessary for scavenging of reactive oxygen species, for protection of membranes, and for modulation of the electron transport chain in the thylakoid membrane [110].

The downregulation of genes related to lipid metabolism, such as those encoding enzymes involved in fatty acid biosynthesis and elongation, synthesis of phospholipids and steroids/squalene and lipid degradation by lipases and lysophospholipases [54] could also be related to reduced growth. However, this could also reflect the regulation of processes involved in altering membrane lipid composition, such as the frequently observed increased unsaturation of fatty acids in membrane lipids in response to low temperatures [3, 4, 135].

The functional group of genes related to hormone metabolism is overrepresented among downregulated genes after both long- and short-term low-temperature treatments. The downregulation of auxin transport and auxin-responsive genes can be interpreted as a contribution to reduced plant growth in response to cold [54, 64]. In addition, downregulation of brassinosteroid (BR)-responsive genes and of genes related to BR biosynthesis could also play a role in controlling the reduction of growth in response to low temperature [54].

All data discussed above were obtained from either leaf tissue or whole *Arabidopsis* rosettes. The cold responses of roots, in contrast, are much less well studied. An investigation of gene expression under various stress conditions including low temperature revealed that roots and leaves display significantly different transcriptional responses [107], with less than 14% of the cold-specific changes shared between roots and leaves. These results are in agreement with the fact that roots are generally considered to have a much lower capacity for cold acclimation than leaves.

11.2.2
Transcriptomic Responses to Low Temperature in Nonmodel Species

Perhaps not very surprisingly, there is a large overlap between genes identified as cold regulated in *Arabidopsis* and in the few studied nonmodel species. A remarkable similarity between genes that have been reported to be cold regulated in *Arabidopsis*

and wheat have, for instance, been reported from a transcriptomic analysis of the cold responses of winter and spring wheat [118]. A possible link between the signal transduction mechanisms during cold acclimation in dicots and monocots was suggested from the increased expression of one of the *PHYA*-like genes in wheat, while the other *PHYA*-like and the *PHYB*-like genes were downregulated. Similarly, phytochromes have been suggested to be involved in the upstream regulation of cold acclimation in *Arabidopsis* [79]. Several recent studies on gene expression of trees under cold conditions show that herbaceous annual and woody perennial plants also share cold acclimation mechanisms and cold-regulated genes [136].

In all investigated species, carbohydrate metabolism-related genes were induced in the cold, including galactinol, raffinose, and sucrose synthases, galactosyltransferases, and genes encoding enzymes involved in starch breakdown [117, 121, 122, 124]. In addition, the downregulated autumn transcriptome in Sitka spruce pointed to a redirection of resources from photosynthesis to stress response during cold acclimation [124], similar to the downregulation of photosynthesis-related genes in *Arabidopsis*, described in more detail above. These common changes will not be discussed separately again for different species.

11.2.2.1 **Cereals: Barley and Wheat**

While in *Arabidopsis* 45% of the investigated genes were cold responsive [54], 28% of the wheat [116] and 25% of the barley [119] transcriptome showed a significant cold regulation. From a comparison of the transcriptomic cold responses of wild-type barley with those in chloroplast-defective mutants, it could be shown that only about 11% of the cold-regulated genes are regulated in a chloroplast-independent way. Significantly, most of the genes that are cold regulated independent of chloroplast function are under CBF control, whereas 67% of the genes regulated in the wild type were not cold-regulated in the mutants and were therefore designated as chloroplast-dependent cold-regulated genes. Strikingly, the effect of the chloroplast on the expression of cold-regulated genes was much larger than the effect of the chloroplast on the transcriptome overall [119].

The largest group of cold-regulated genes in the wild type encodes ribosomal proteins, while the mutants with inactive chloroplasts show constitutively active photooxidative stress signaling pathways and a disruption of low-temperature signaling pathways [119].

The transcriptomic cold response of barley cv. Morex was investigated over 33 days and 2331 genes were identified as low-temperature responsive [120]. These genes are, for instance, involved in signaling cascades and transcriptional control, in biogenesis of cellular components, cell cycle, and DNA processing, protein modification and destination, cellular transport, antioxidant defense, and metabolism of osmoprotectants and lipids.

In wheat 303 upregulated and 378 downregulated genes were identified in the moderately freezing-sensitive cultivar CS and in two CS-derived substitution lines in which chromosome 5A originated from either a more freezing-tolerant (Ch) or a more freezing-sensitive cultivar (Tsp) [117]. In this case, alterations of transcript levels during the first days of low-temperature treatment were larger than during later

stages of cold, similar to the situation in *Arabidopsis* [54]. Upregulated genes encoded, for example, enzymes of proline and ethylene biosynthesis, transcription factors, and known stress-related proteins, and enzymes involved in secondary and polyamine metabolism, whereas downregulated genes included, among others, those encoding enzymes of lipid, protein, and antioxidant metabolism and proteins involved in photosynthesis, respiration, transcription, and transport processes.

11.2.2.2 **Perennial Shrubs: Blueberry**

In floral buds from blueberry transcriptomic analyses were performed under both field and controlled growth chamber conditions [121]. In addition to many genes that had already been identified as cold regulated in *Arabidopsis*, several other genes were identified, including genes encoding an auxin-repressed protein, the protein kinase PINOID, pectate lyase-like protein, and *S*-adenosylmethionine decarboxylase proenzyme. Interestingly, under growth chamber conditions more genes were upregulated in the cold than under field conditions, including stress tolerance genes, genes encoding enzymes of the glycolytic and TCA cycle, and genes associated with protein biosynthesis. Some of the genes exclusively induced under field conditions were related to light stress [121].

11.2.2.3 **Trees: Poplar, Pine, and Spruce**

During the induction of cambial dormancy in *Populus tremula*, a massive remodeling of the transcriptome and a significant reduction in the complexity of the cambial transcriptome occur [122]. The establishment of dormancy is accompanied by an increase in freezing tolerance, as observed at the transcript level by an induction of genes encoding, for example, LEA proteins, osmotin, chitinases, and enzymes involved in sucrose and raffinose biosynthesis and in oxidative stress responses.

Scots pine (*Pinus sylvestris*) seedlings were analyzed for transcript abundance in apical buds under different conditions (three field experiments, two growth chambers, two seasons, and two plant ages) using a cDNA microarray containing about 1500 expressed sequence tags (ESTs) from buds of cold-treated Scots pine seedlings [123]. Samples from three locations in Sweden, Denmark, and Scotland and four provenances were compared. Correlations between physiological parameters, environmental conditions, and gene expression could be shown and led to the identification of potential marker genes for freezing tolerance, including genes encoding antifreeze and LEA proteins, metabolic enzymes, and other stress- or ABA-induced proteins.

Sitka spruce (*Picea sitchensis*) was monitored for gene expression within and among populations comparing late summer and early winter when cold acclimation is initiated [124]. Among the 1257 and 967 genes that were at least twofold up- or downregulated, respectively, many are already well known from *Arabidopsis*. Possible members of signal transduction pathways include genes encoding calcium-binding proteins, such as calmodulins, calcineurin B-like (CBL), and CBL-interacting protein kinases (CIPKs) and the Ca^{2+}/H^{+} antiporter *CAX1*. However, 549 upregulated and 387 downregulated genes have no homology to any *Arabidopsis* genes, indicating also more specific cold responses in this conifer.

11.2.3
Influence of Deacclimation on Plant Gene Expression

Only little effort has been directed toward the analysis of gene expression during deacclimation, that is, the loss of cold acclimation when plants are shifted back to nonacclimating conditions after low-temperature treatment. At a physiological level, it has been shown that *Arabidopsis* and other plants rapidly lose freezing tolerance when transferred back to warm growth conditions [137, 138]. In *Arabidopsis* plants grown on agar plates, 292 genes were identified as upregulated and 320 as downregulated during deacclimation for 1–24 h [108]. They belong to various functional groups, such as transcription factors, detoxification, biosynthesis or catabolism of amino acids, cell wall-related proteins, and proteins involved in photosynthesis or carbohydrate metabolism. Differentially regulated genes were clustered and a large group of genes that were found to be strongly downregulated during deacclimation were also strongly upregulated during cold acclimation. The fact that most of the already known cold-inducible genes belong to this cluster strengthens the hypothesis that these genes might be involved in key metabolic pathways important for cold acclimation. Transcription factors upregulated during deacclimation are obvious candidates for the downregulation of such cold upregulated genes.

11.2.4
Profiling Gene Expression during Cold Acclimation Using SAGE Technology

In addition to the numerous microarray studies discussed above, the SAGE (serial analysis of gene expression) technology [139] has been used only in a few studies and exclusively in *Arabidopsis*, to analyze gene expression during cold acclimation [140–143]. Since SAGE is a sequencing-based technology, it is independent of previously established gene models. It can, therefore, be used to detect the involvement of novel genes, different splice variants, or antisense transcripts in a biological process.

In a comparison of SAGE libraries from 3 days cold-treated leaves with a nonacclimated control, 272 differentially expressed genes, 190 of them highly upregulated in cold-treated leaves, were found [141]. Upregulated genes encode proteins involved in cell rescue, defense, cell death, aging, protein synthesis, metabolism, transport facilitation, lipid metabolism, and protein destination, whereas downregulated genes encode proteins involved in photosynthesis and photorespiration. Examples of the most strongly induced transcripts (*COR15a*, *RD29A/COR78*, *LTI130*, alcohol dehydrogenase, β-amylase, chalcone synthase, and a lipid transfer protein) show that SAGE technology recognizes the same cold-induced genes as microarray technology. However, from 134 genes with at least 10-fold changes in expression, 87 were newly identified as cold-stress-related genes, reflecting the great potential of SAGE to identify novel stress-related genes.

In a time course experiment from the perception of cold to the acquisition of freezing tolerance, using five SAGE libraries from 30 min up to 1 week of low-temperature treatment, 920 low-temperature responsive genes were identified [143].

Only 24% of these genes were identical to those previously identified using microarrays. The identification of novel genes independent of CBF and ABA regulation was possible and pointed to complementary signaling pathways. In addition, alternative transcript processing, such as retained introns, modified exon structure, and polymorphic UTR sequences, was identified as important for the plasticity of the stress-induced transcriptome.

Recently, the LongSAGE technology [144] was used to analyze the initial events involved in signal transduction after cold exposure (0 °C, 1 h) in *Arabidopsis* leaves [140]. In comparison to conventional SAGE tags, the longer tags from this technology increase the chances for the identification of novel genes. By comparing control with cold-treated leaves, 315 differentially expressed genes could be identified that, however, mostly corresponded to the expected changes in gene expression. Interestingly, differential expression of miRNAs was detected, most of which were downregulated. The functional significance of these changes in miRNA abundance in the early responses of leaves to cold stress remains to be elucidated.

In addition to leaf tissue, SAGE technology was also used to investigate gene expression in *Arabidopsis* pollen at low temperature [142]. Comparing a SAGE library from pollen with a library from cold-treated leaves showed that most of the highly abundant genes responsible for pollen function are not greatly affected by a cold stress at 0 °C for 72 h, whereas seed production was reduced. Most of those genes that are cold induced in vegetative tissue showed unchanged expression levels or only a weak induction in pollen under cold stress. This might explain the high sensitivity of *Arabidopsis* pollen to low temperature.

11.2.5
Effects of Low Temperature on Circadian Clock-Regulated Gene Expression

A first suggestion that the circadian clock is involved in plant cold responses came from the upregulation of the transcript from the *GIGANTEA* gene, involved in flowering time regulation and circadian clock function [58]. *GIGANTEA* was also found to be fivefold induced in the autumn transcriptome of Sitka spruce following natural cold acclimation [124]. Kreps *et al.* [107] observed a stress response for 68% of the known circadian controlled genes that supported the role of the circadian clock in stress anticipation and activation of cold stress pathways. A strong upregulation of *APRR1* (*pseudo-response regulator 1*), an essential component of the central oscillator of the circadian clock in *Arabidopsis*, after exposure to low temperature was an additional indication for the involvement of the circadian clock in the cold response [64].

In a microarrays analysis of diurnal (light/dark) and circadian (constant light) time courses under nonacclimating and acclimating conditions in *Arabidopsis*, it could be shown that under diurnal conditions cold reduces the amplitude of the clock cycles and disrupts the cycling of some of the circadian output genes, while in continuous light all cycles become arrhythmic [115, 145]. Similarly, circadian clock function is disrupted in chestnut during winter dormancy [146, 147]. Expression analysis of approximately 1900 genes encoding transcription factors of *Arabidopsis* by quanti-

tative real-time RT-PCR [148] showed diurnal gating with a stronger cold induction for 75% of the cold-induced transcription factor genes and a higher absolute transcript abundance for most of them in the morning [145]. These findings confirmed and massively extended previous results [149] showing gating of the cold induction of the *CBF1, 2,* and 3 and *RAV1* and *ZAT12* genes. These strong circadian effects on the expression patterns of cold-regulated genes are contrary to results from AtGenExpress [106] where, however, very different culture conditions were used. Interactions between stress and diurnal regulation of gene expression not only influence the cold-responsive transcriptome but also gene expression in response to drought stress [150], reflecting the importance of time-of-day on the identification of stress-responsive genes and should be taken into account in future gene expression analysis experiments.

11.2.6
Using Natural Genetic Variation to Identify Genes Important for Cold Acclimation and Freezing Tolerance

Several studies in *Arabidopsis* have shown a large variability in acclimated freezing tolerance between different accessions that follows a clear latitudinal cline with increasing freezing tolerance in accessions from more northern habitats [25, 38, 40]. Likewise, cultivar differences in freezing tolerance have been reported in various crop plants (e.g., [31, 32, 35]). Comparisons of transcriptomic cold responses of accessions, cultivars, or populations differing in freezing tolerance have been published for *Arabidopsis* [25, 151], wheat [116–118], and Sitka spruce [124].

In *Arabidopsis*, an investigation of nine accessions that differed widely in freezing tolerance [25] found differential transcriptional regulation between accessions after exposure to low temperature. Both the number of significant changes in gene expression during cold acclimation and the amplitude of those changes were significantly correlated with the ability of the accessions to increase their freezing tolerance. In particular, the downregulation of photosynthesis-related genes and the upregulation of genes encoding enzymes of the flavonoid biosynthesis pathway were correlated with improved freezing tolerance. Furthermore, the expression of 32 genes encoding transcription factors, including the *CBF*s, correlated positively, while the expression of 24 such genes correlated negatively with freezing tolerance, providing candidates for key transcriptional regulators of cold acclimation.

In a study of 10 *Arabidopsis* accessions from a wide range of latitudes, the impact of low temperature was investigated with special consideration of the adaptive significance of regulated genes [151]. Genes with high plasticity in their expression patterns exhibited higher among-ecotype variation that was also strongly correlated with the habitat temperature at the geographic origin of the ecotypes. Forty-three genes with significant expression plasticity combined with adaptive value were found.

Similar to *Arabidopsis*, in wheat a freezing tolerant cultivar showed 1.5 times as many differentially expressed genes during cold acclimation than a sensitive cultivar [117]. Over 450 genes were differentially cold regulated between the highly freezing tolerant winter wheat cultivar CDC Clair and the less tolerant spring cultivar

Quantum, including 130 genes involved in gene regulation or signaling [118]. Within the first hours of cold acclimation, more genes were upregulated in the sensitive than in the tolerant cultivar, whereas after 2 weeks of acclimation the response was reversed, with the winter wheat finally showing more up- and downregulated genes than the spring cultivar. Similarly, in another pair of wheat cultivars (Glenlea; sensitive and Norstar; tolerant), 65 cold-regulated genes were found differentially regulated after 1–36 days of cold acclimation [116].

In a comparison between three populations of Sitka spruce (*Picea sitchensis*) with a central population from British Columbia and northern and southern peripheral populations from Alaska and California that markedly differed in freezing tolerance, a substantial differential gene expression among populations was reported [124]. In the northern and central populations, 326 genes showed higher expression than in the southern population. Among these genes were several that encode proteins that are presumably directly involved in freezing tolerance and also several that encode regulators of gene expression. In the light of future climate change predictions, the search for markers characterizing the local adaptation of a species will become increasingly important for molecular breeding especially of long-lived woody plants [124].

11.3
Metabolomic Studies of Plant Cold Acclimation

Recently developed metabolic profiling tools such as GC-MS and LC-MS methods have been used to study global changes in metabolite content of *Arabidopsis* in response to low temperature and have led to a detailed description of the massive metabolic reprogramming occurring after a shift of plants from ambient conditions to cold [25, 57, 59, 131, 152]. The functional role of these complex metabolic changes during cold acclimation is not clear. However, it can be assumed that they are in part related to the strong reduction in growth rate at low temperatures, which is an active regulatory process rather than a passive result of temperature reduction [72]. Equally, it is very likely that many molecules synthesized during cold acclimation contribute directly to increased freezing tolerance. They could have cryoprotective effects, such as soluble sugars, sugar alcohols, and some amino acids. In addition, some are likely to function, for example, as antioxidants.

To obtain a broader overview of the influence of cold acclimation on metabolic regulation, we recently performed metabolic network analysis using time course GC-MS metabolic profiling data [115]. Figure 11.1 presents the metabolic networks reconstructed from these data for *Arabidopsis* plants grown either at 20 °C or at 4 °C. In these undirected metabolic networks, the nodes represent metabolites and the edges (i.e., the lines connecting the nodes) represent highly significant correlations in the amounts of the two respective metabolites over time. Visual comparison of the two networks indicates significant differences. Topological network analysis indicates a higher network density with a higher number of edges (i.e., connections between metabolites) at 4 °C (205 edges) than at 20 °C (121 edges). Also, the number

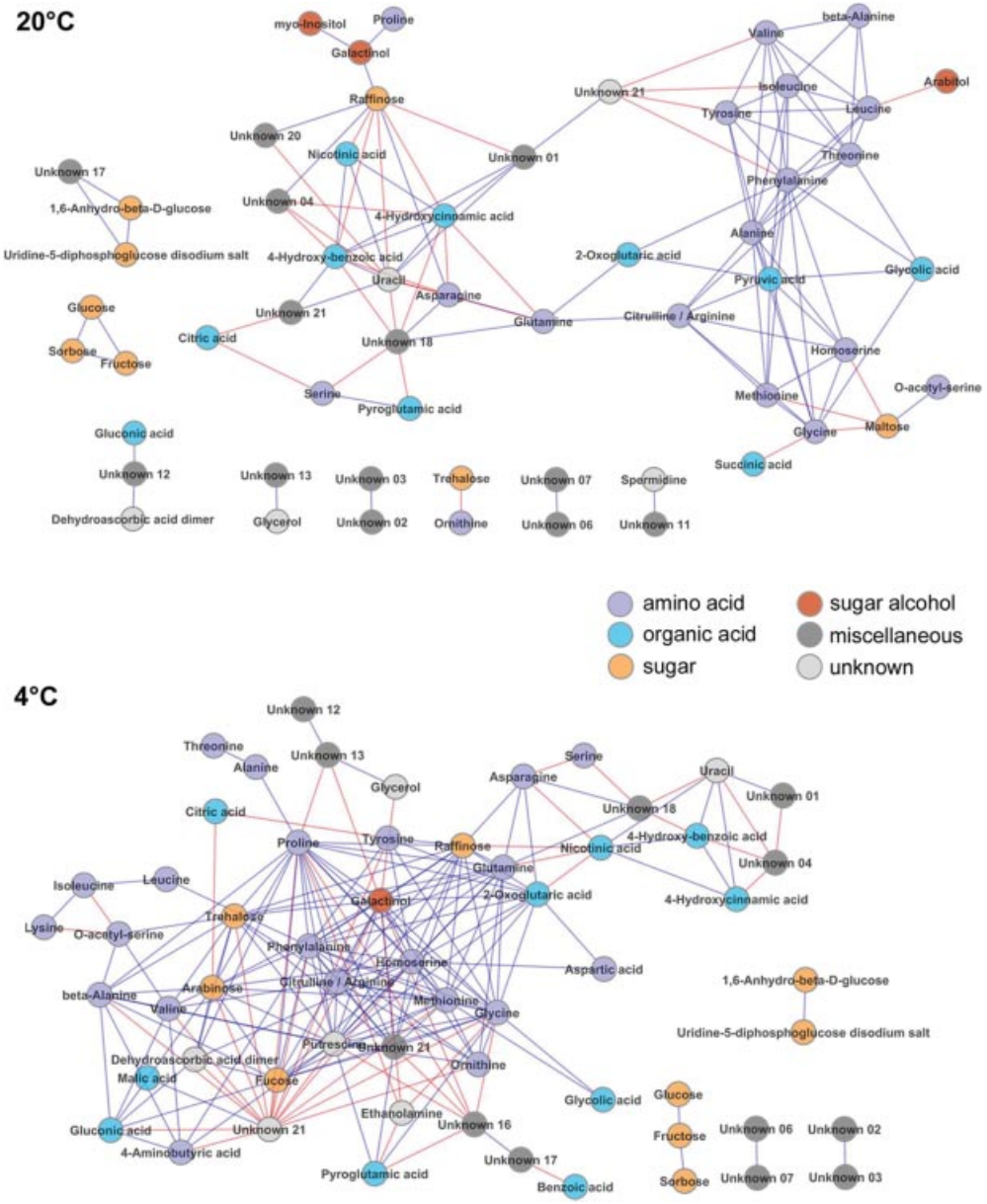

Figure 11.1 Effect of low temperature on the metabolite–metabolite correlation network. Networks were constructed on the basis of Spearman metabolite–metabolite correlations, with nodes (colored dots) representing the measured metabolites. Undirected edges (lines) indicate highly significant (Bonferroni corrected p-value < 0.001, corresponding to a correlation coefficient R of about 0.54 for the given sample size) positive (blue) and negative (red) pairwise correlations between metabolite pool sizes. Massive metabolic changes occurring at low temperature are indicated by the higher number of significant correlations observed at 4 °C, compared to 20 °C. These additional correlations mainly involve molecules acting as compatible solutes. Reproduced from Ref. [115].

of connected pairs significantly increased from 1434 at 20 °C to 2462 at 4 °C [115]. These and other parameters clearly indicate that the metabolic reprogramming during cold acclimation is a tightly regulated process and not simply the result of metabolic dysfunction due to low-temperature stress.

The three cold-induced CBF transcription factors have a prominent role in this metabolic reconfiguration, with around 80% overlap between metabolic responses to the constitutive overexpression of *CBF3* and cold acclimation [57]. However, it is widely recognized that cold acclimation is a highly complex process not only at the level of gene expression but also at the metabolic level. Consequently, it could be shown that metabolic reconfiguration at low temperature is strongly influenced by stress duration [152] and the developmental state of the plants [152, 153]. Another factor contributing to the complexity of the plant response to low temperature is the widely recognized diurnal regulation of many metabolic pathways in plants, such as starch and sugar metabolism [154, 155]. This can lead to the superposition of diurnal changes in metabolite pools on changes induced by low temperature [131, 152]. Therefore, both diurnal and circadian regulation of metabolism can affect the identification of cold-responsive metabolites [115] similar to the situation with transcripts described above [115, 145]. Nevertheless, many metabolic pathways have clearly been shown to be influenced by cold acclimation.

11.3.1 Primary Metabolism

The reconfiguration of the metabolome of *Arabidopsis* during cold acclimation involves dramatic changes in central metabolism. These changes include modifications in the pool sizes of amino acids such as alanine, citrulline, glutamine, and *O*-acetylserine [57]. Moreover, due to the increase in aspartate, ornithine, and citrulline, an upregulation of the urea cycle has been suggested and the increased levels of several intermediates of the TCA cycle, such as α-ketoglutarate, fumarate, malate, and citrate, also support an upregulation of this central metabolic process [131].

Changes in the size of hexose phosphate pools and the free hexose pools that occur at low temperature suggest a modification of carbon metabolism in photosynthetic tissues toward the accumulation of a variety of sugar phosphates and simple sugars [156]. In particular, photosynthesis is negatively affected by low temperature, leading to an accumulation of phosphorylated intermediates with a concomitant decrease in inorganic phosphate levels [157, 158]. Changes in central carbohydrate metabolism are also subject to temporal regulation during acclimation. For instance, at early stages of low-temperature exposure, compounds such as maltose, fructose, fructose-6-phosphate, glucose, glucose-6-phosphate, and glycerol-3-phosphate seem to have a relevant role, while after longer acclimation times the accumulation of sorbitol and galactinol becomes significant [152].

The analysis of changes in approximately 200 metabolites by GC-MS during cold acclimation in nine *Arabidopsis* accessions has not revealed a clear correlation between global metabolic changes and acclimation capacity. Nevertheless, the pool

sizes of metabolites such as glucose, fructose, galactinol, raffinose, xylose, and sucrose were significantly correlated with acclimated freezing tolerance, reflecting the importance of carbohydrate metabolism [25]. Similar correlations or a lack thereof has previously been reported in many plant species (see Ref. [1] for a review of the older literature in this field), casting doubt on the functional relevance of particular sugars for freezing tolerance. More recent work on *Arabidopsis* suggested that it may not be a specific sugar that is important for plant freezing tolerance, but that sugars may constitute a highly redundant cryoprotective system [105, 159]. However, an increase in total sugar content is also not sufficient to significantly increase leaf freezing tolerance [20]. These data suggest that several metabolites may act together with other factors such as cold-induced proteins [2] to increase cellular freezing tolerance. Metabolite profiling together with more sophisticated statistical data analysis tools is uniquely capable of providing solutions for such complex problems. For a panel of *Arabidopsis* genotypes (accessions and F1 crosses), this approach led to the identification of a small group of metabolites that allowed to predict both acclimated and nonacclimated freezing tolerance with high accuracy [160]. This group contained not only sugars, amino acids, and organic acids but also some unidentified metabolites. The latter finding indicates that there may still be important metabolites even in a well-studied species such as *Arabidopsis* that play important physiological roles but have not been characterized yet.

In addition, such analyses can provide evidence for novel functions of already well-characterized metabolites. For example, it was found that increased freezing tolerance during cold acclimation is preceded by maltose accumulation [161]. Maltose, as a starch degradation product, has usually not been considered a compatible solute, although it is able to stabilize membranes during desiccation [162] and was shown to contribute, either alone or by increasing other soluble sugars, to the protection of photosynthetic electron transport during freezing stress [161]. Moreover, maltose could act as a carbon source as the process of cold acclimation requires considerable energy supply. Experimental data point to a key role for starch breakdown during the process of cold acclimation [163]. This finding is well validated by the demonstration that two mutants that are impaired in starch breakdown have reduced ability to cold acclimate [161, 164].

11.3.2 Secondary Metabolism

While there is large interest in the biosynthesis and properties of flavonoids and isoflavonoids in plants, both from the perspective of plant biology and that of their potential medical applications, very little clear information on the specific roles of any of these compounds is available (see Ref. [165] for a recent review). The effect of low temperatures on secondary metabolism has been widely recognized and flavonoid biosynthesis is strongly upregulated during cold acclimation [25, 105, 114, 166].

Flavonoids constitute a family of aromatic compounds synthesized from L-phenylalanine and malonyl-coenzyme A, through the phenylpropanoid pathway. Several of the genes encoding key enzymes in this pathway have been identified as cold

responsive, including, for example, L-phenylalanine ammonialyase (PAL) and chalcone synthase (CHS) [133, 167]. Flavonoids comprise the chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins. Anthocyanins are strongly accumulated in response to different environmental factors, such as low temperature, drought, and high light. One of the main properties of flavonoids is their ability to absorb ultraviolet (UV) light and together with the fact that they are often present in tissues highly exposed to light (epidermal cell layers in leaves, pollen, and apical meristem), it has been proposed that the main role of flavonoids is protection against UV-light [133]. Cold acclimation has a major influence on the amount and composition of flavonols [105]. Even when cold stress induces the synthesis of anthocyanins and flavonoids, glucosinolates, terpenoids, and phenylpropanoids [113], the biological significance of this remains to be demonstrated. However, the positive correlation between flavonol content and leaf freezing tolerance strongly suggests that flavonols can play a role in plant acclimation and freezing tolerance [105]. Putative physiological functions suggested for flavonoids during cold and freezing include scavenging of reactive oxygen species [132] or their function as a carbon sink during cold stress [168, 169]. In addition, flavonols may also have direct effects on membrane stability. Some flavonols can partition into the lipid phase of membranes [170]. Under freezing conditions, when a large part of the water is removed from the cells to intercellular ice crystals, it can be expected that amphiphiles such as flavonoids will partition even more strongly into the hydrophobic phase of membranes [171]. While there is as yet no experimental evidence for a membrane stabilizing function of plant flavonoids, it has been shown that the glucosylated phenol arbutin (4-hydroxyphenol-β-D-glucopyranoside) that is found in some extremely frost- or desiccation-tolerant plants is able to specifically stabilize membranes that contain nonbilayer lipids [172, 173]. Similar functions could be envisaged for flavonoids, but this needs to be experimentally tested.

11.3.3 Lipid Metabolism

A decrease in temperature reduces the fluidity of membranes and can in extreme cases lead to a phase transition of the lipids from the liquid-crystalline to the gel state (thermotropic phase transition). Owing to the crystallization of ice, freezing also leads to dehydration, which further increases the lipid-phase transition temperature and the propensity of the lipids to enter the gel state (lyotropic-phase transition). Such a phase transition can cause the segregation and aggregation of membrane proteins into a remaining liquid–crystalline phase with the consequent inactivation of the proteins. Furthermore, membranes can become leaky at the phase boundaries due to nonideal packing, leading to the loss of semipermeability of cellular membranes and loss of compartimentation [174]. In addition, chilling-sensitive plants and cyanobacteria show an inhibition of photosynthesis [175] that could be related by mutational and transgenic approaches to the ratio of saturated to unsaturated phosphatidylglycerol in chloroplast membranes [176–178]. In chilling-tolerant plants such as *Arabidopsis*, a high content of polyunsaturated lipids is required for growth and

development at low temperatures (e.g., [135, 179, 180]) and cereals increase the content of unsaturated fatty acids in their membrane lipids during cold acclimation [4]. Fatty acid analysis has also revealed increases in polyunsaturated molecules in *Arabidopsis* in response to cold (C18 : 2; C18 : 3; and C16 : 3), while less unsaturated fatty acids decrease in their content (C16 : 2 and C16 : 1) [114]. Cold acclimation and mild freezing (i.e., subzero acclimation) can also increase the proportion of bilayer lipids, such as phosphatidylcholine and digalactosyldiacylglycerol, and decrease the proportion of nonbilayer lipids such as cerebrosides, phosphatidylethanolamine, and monogalactosyldiacylglycerol (e.g., [181, 182]), thus reducing the incidence of fatal interbilayer fusion events.

11.3.4
Sulfur and Nitrogen Metabolism

The pool size of *O*-acetylserine (OAS) in *Arabidopsis* leaves is strongly increased in response to low temperature [115]. OAS, a direct precursor of cysteine, is synthesized by the activity of serine acetyltransferase (SAT) from serine. OAS is then converted to cysteine by *O*-acetylserine (thiol) lyase (OASTL). It has been proposed that OAS acts as part of the regulatory network signaling metabolic demand for sulfur-containing compounds [183]. Glutathione is the most abundant thiol compound in plant cells, playing a major role as an antioxidant during stress [183]. It is, therefore, possible that the increase in OAS levels during cold acclimation is related to an increased freezing tolerance through the antioxidant defense system. Consistent with this, many genes encoding enzymes involved in ROS scavenging are also cold responsive.

Nitrogen metabolism plays a key role in plant physiology. Glutamic acid and glutamine act as ammonium donors in the synthesis of all other amino acids. Aspartic acid and asparagine further act as active ammonium donors or transport/storage compounds, respectively [184]. Both glutamine and asparagine are accumulated at low temperatures [57]. GABA (γ-aminobutyric acid) levels highly increase after plants are exposed to cold. GABA is synthesized in the cytosol from glutamine and is an important amine-containing metabolite with cryoprotective properties and with a putative role in stress signaling [185–187].

11.4
Both Transcriptional and Posttranscriptional Regulation of Metabolism are Important During Cold Acclimation

As already briefly discussed in Section 11.1, interpretation of changes observed in transcript abundance during cold acclimation in profiling studies assumes that mRNA amounts are the limiting factor that determines physiological responses. We will briefly discuss two recent studies that have shed some light on the limitations of this assumption.

One study used expression profiling combined with enzyme activity measurements and metabolite profiling to investigate the responses of *Arabidopsis* 6 and 78 h after transfer from 20 °C to a progressively lower temperature between 17 and 8 °C [114].

Transcript abundances showed stronger changes as the temperature difference increased and the changes were generally larger after 6 h than after 78 h. Changes in enzyme activities and metabolite pool sizes showed a similar dependence on temperature, but here the changes were larger after 78 h than after 6 h. The correlations between the abundance of specific transcripts and the encoded enzyme activities were very poor after 6 h of cold treatment and significantly improved after 78 h. However, also after 78 h of cold treatment, a considerable part of the changes in gene expression and enzyme activity were unrelated, pointing to important contributions of translational regulation and/or protein stability to the observed changes in enzyme activities. In addition, discrepancies between changes in extractable enzyme activity and changes in metabolite pool sizes were noted, indicating the importance of further regulatory mechanisms that influence enzyme activity *in vivo*. Interestingly, this study suggests that a large part of the transcriptional responses to small changes in temperature were driven by the observed increases in *CBF* expression.

In another study [115], transcripts and primary metabolites were quantified during diurnal time courses at either 20 °C or at 4 °C. These data provide a comprehensive

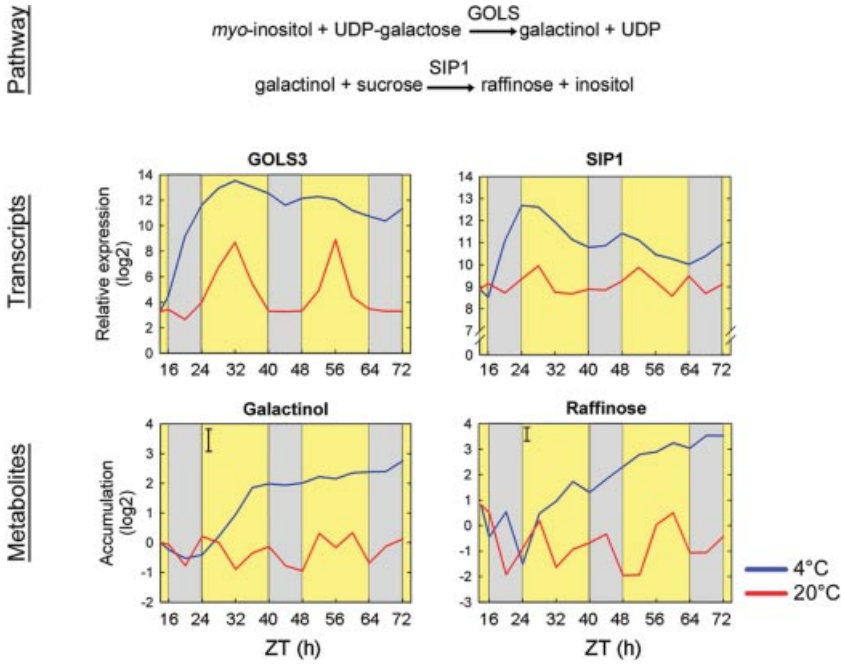

Figure 11.2 Coordinated transcriptional regulation of the raffinose biosynthesis pathway. Summary of the metabolic pathway, transcript, and metabolite profiles of the raffinose biosynthesis pathway. For transcripts, relative expression (log2) estimates from Affymetrix ATH1 microarrays are indicated. Metabolite accumulation (log2) was measured by GC-TOF-MS and it is expressed as the normalized mean peak apex intensities from five biological replicates. GOLS3: galactinol synthase 3 (At1g09350); SIP1: raffinose synthase (At5g40390). Reproduced from Ref. [115].

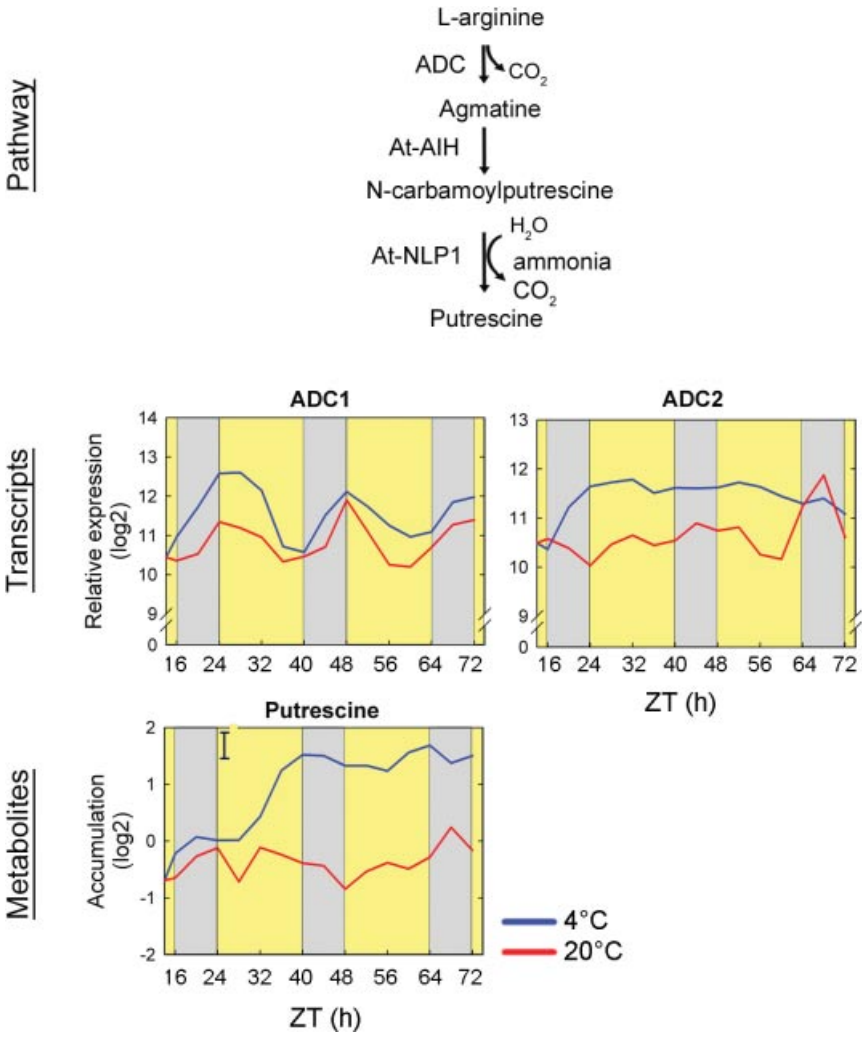

Figure 11.3 Coordinated transcriptional regulation of the polyamine biosynthesis pathway. Summary of the metabolic pathway, transcript, and metabolite profiles of the polyamine biosynthesis pathway leading to putrescine. For transcripts, relative expression (log2) estimates from Affymetrix ATH1 microarrays are indicated. Metabolite accumulation (log2) was measured by GC-TOF-MS and it is expressed as the normalized mean peak apex intensities from five biological replicates. ADC: arginine decarboxylase 1 (At2g16500) or 2 (At4g34710); At-AIH: agmatine iminohydrolase; At-NLP1: *N*-carbamoylputrescine amidohydrolase. Reproduced from Ref. [115].

analysis of the diurnal regulation of both gene expression and primary metabolism that interact with the cold regulation of both processes. A previous study had shown that the diurnal cycling of clock genes becomes strongly dampened in the cold [145] and this extends to the diurnal cycling of metabolites. However, 80% of those

metabolites that show cycles at 20 °C still do so at 4 °C [115]. The transcripts encoding enzymes for biosynthesis of well-known cold-induced metabolites such as raffinose and putrescine also cycled at 20 °C and diurnal cycling was maintained in the cold (Figures 11.2 and 11.3). Also, in both cases, increased transcript abundance preceded the cold induction of the related metabolites, in agreement with a predominantly transcriptional regulation of these pathways.

Interestingly, in the raffinose pathway *GOLS3* expression was rapidly induced and stayed high until the end of the experiment, while *SIP1* was also rapidly induced, but here the induction was transient and the expression level approached control values after 2 days at 4 °C (Figure 11.2). Nevertheless, a continuous increase in raffinose content was observed indicating additional regulatory mechanisms even in this case. Another regulatory mechanism is illustrated in Figure 11.3. In *Arabidopsis*, two genes encode the enzyme arginine decarboxylase and while *ADC1* expression is only slightly influenced by cold, *ADC2* is more strongly upregulated, pointing to the specific role of only one of the genes in stress adaptation. In addition, in many cases no correlation between changes in transcript abundance and metabolite pool sizes could be observed [115], emphasizing the importance of translational and posttranslational mechanisms in metabolic regulation.

References

1 Steponkus, P.L. (1984) Role of the plasma membrane in freezing injury and cold acclimation. *Annu. Rev. Plant Physiol.*, **35**, 543–584.

2 Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 571–599.

3 Xin, Z. and Browse, J. (2000) Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant Cell Environ.*, **23**, 893–902.

4 Steponkus, P.L., Uemura, M., and Webb, M.S. (1993) A contrast of the cryostability of the plasma membrane of winter rye and spring oat: two species that widely differ in their freezing tolerance and plasma membrane lipid composition, in *Advances in Low-Temperature Biology* (ed. P.L. Steponkus), JAI Press, London, England, pp. 211–312.

5 Ehlert, B. and Hincha, D.K. (2008) Chlorophyll fluorescence imaging accurately quantifies freezing damage and cold acclimation responses in *Arabidopsis* leaves. *Plant Methods*, **4**, 12.

6 Hincha, D.K., Höfner, R., Schwab, K.B., Heber, U., and Schmitt, J.M. (1987) Membrane rupture is the common cause of damage to chloroplast membranes in leaves injured by freezing or excessive wilting. *Plant Physiol.*, **83**, 251–253.

7 Hincha, D.K. and Schmitt, J.M. (1992) Freeze–thaw injury and cryoprotection of thylakoid membranes, in *Water and Life* (eds G.N. Somero, C.B. Osmond, and C.L. Bolis), Springer, Berlin, pp. 316–337.

8 Stone, J.M., Palta, J.P., Bamberg, J.B., Weiss, L.S., and Harbage, J.F. (1993) Inheritance of freezing resistance in tuber-bearing *Solanum* species: evidence for independent genetic control of nonacclimated freezing tolerance and cold acclimation capacity. *Proc. Natl. Acad. Sci. USA*, **90**, 7869–7873.

9 Tsarouhas, V., Gullberg, U., and Lagercrantz, U. (2004) Mapping of quantitative trait loci (QTLs) affecting autumn freezing resistance and phenology in *Salix*. *Theor. Appl. Genet.*, **108**, 1335–1342.

10 Kole, C., Thormann, C.E., Karlsson, B.H., Palta, J.P., Gaffney, P., Yandell, B., and Osborn, T.C. (2002) Comparative

mapping of loci controlling winter survival and related traits in oilseed *Brassica rapa* and *B. napus*. *Mol. Breed.*, **9**, 201–210.

11 Teutonico, R.A., Yandell, B., Satagopan, J.M., Ferreira, M.E., Palta, J.P., and Osborn, T.C. (1995) Genetic analysis and mapping of genes controlling freezing tolerance in oilseed *Brassica*. *Mol. Breed.*, **1**, 329–339.

12 Herman, E.M., Rotter, K., Premakumar, R., Elwinger, G., Bae, R., Ehler-King, L., Chen, S., and Livingston, D.P. (2006) Additional freeze hardiness in wheat acquired by exposure to −3 °C is associated with extensive physiological, morphological, and molecular changes. *J. Exp. Bot.*, **57**, 3601–3618.

13 Livingston, D.P. (1996) The second phase of cold hardening: freezing tolerance and fructan isomer changes in winter cereal crowns. *Crop. Sci.*, **36**, 1568–1573.

14 Livingston, D.P. and Henson, C.A. (1998) Apoplastic sugars, fructans, fructan exohydrolase, and invertase in winter oat: responses to second-phase cold hardening. *Plant Physiol.*, **116**, 403–408.

15 Livingston, D.P., Premakumar, R., and Tallury, S.P. (2005) Carbohydrate concentrations in crown fractions from winter oat during hardening at sub-zero temperatures. *Ann. Bot.*, **96**, 331–335.

16 Olien, C.R. and Lester, G.E. (1985) Freeze-induced changes in soluble carbohydrates of rye. *Crop Sci.*, **25**, 288–290.

17 Le, M.Q., Engelsberger, W.R., and Hincha, D.K. (2008) Natural genetic variation in acclimation capacity at sub-zero temperatures after cold acclimation at 4 °C in different *Arabidopsis thaliana* accessions. *Cryobiology*, **57**, 104–112.

18 Livingston, D.P., Van, K., Premakumar, R., Tallury, S.P., and Herman, E.M. (2007) Using *Arabidopsis thaliana* as a model to study subzero acclimation in small grains. *Cryobiology*, **54**, 154–163.

19 Close, T.J., Wanamaker, S.I., Caldo, R.A., Turner, S.M., Ashlock, D.A., Dickerson, J.A., Wing, R.A., Muehlbauer, G.J., Kleinhofs, A., and Wise, R.P. (2004) A new resource for cereal genomics: 22 K barley GeneChip comes of age. *Plant Physiol.*, **134**, 960–968.

20 Hincha, D.K., Sonnewald, U., Willmitzer, L., and Schmitt, J.M. (1996) The role of sugar accumulation in leaf frost hardiness – investigations with transgenic tobacco expressing a bacterial pyrophosphatase or a yeast invertase gene. *J. Plant Physiol.*, **147**, 604–610.

21 Hirsh, A.G., Williams, R.J., and Meryman, H.T. (1985) A novel method of natural cryoprotection. *Plant Physiol.*, **79**, 41–56.

22 Rütten, D. and Santarius, K.A. (1988) Cold acclimation of *Ilex aquifolium* under natural conditions with special regard to the photosynthetic apparatus. *Physiol. Plant.*, **72**, 807–815.

23 Strand, M. and Öquist, G. (1988) Effects of frost hardening, dehardening and freezing stress on *in vivo* chlorophyll fluorescence of seedlings of Scots pine (*Pinus sylvestris* L.). *Plant Cell Environ.*, **11**, 231–238.

24 Fennell, A., Li, P.H., and Markhart, A.H. (1990) Influence of air and soil temperature on water relations and freezing tolerance of spinach (*Spinacia oleracea*). *Physiol. Plant.*, **78**, 51–56.

25 Hannah, M.A., Wiese, D., Freund, S., Fiehn, O., Heyer, A.G., and Hincha, D.K. (2006) Natural genetic variation of freezing tolerance in *Arabidopsis*. *Plant Physiol.*, **142**, 98–112.

26 Rumich-Bayer, S. and Krause, G.H. (1986) Freezing damage and frost tolerance of the photosynthetic apparatus studied with isolated mesophyll protoplasts of *Valerianella locusta* L. *Photosynth. Res.*, **8**, 161–174.

27 Yelenosky, G. and Guy, C.L. (1989) Freezing tolerance of citrus, spinach, and petunia leaf tissue. *Plant Physiol.*, **89**, 444–451.

28 Li, C., Puhakainen, T., Welling, A., Viherä-Aarnio, A., Ernstsen, A., Junttila, O., Heino, P., and Palva, E.T. (2002) Cold acclimation in silver birch (*Betula pendula*). Development of freezing tolerance in different tissues and climatic ecotypes. *Physiol. Plant.*, **116**, 478–488.

29 Puhakainen, T., Li, C., Boije-Malm, M., Kangasjärvi, J., Heino, P., and Palva, E.T. (2004) Short-day potentiation of low

temperature-induced gene expression of a C-repeat-binding factor-controlled gene during cold acclimation in silver birch. *Plant Physiol.*, **136**, 4299–4307.

30 Alonso-Blanco, C., Gomez-Mena, C., Llorente, F., Koornneef, M., Salinas, J., and Martinez-Zapater, J.M. (2005) Genetic and molecular analyses of natural variation indicate *CBF2* as a candidate gene for underlying a freezing tolerance quantitative trait locus in *Arabidopsis*. *Plant Physiol.*, **139**, 1304–1312.

31 Baga, M., Chodaparambil, S.V., Limin, A.E., Pecar, M., Fowler, D.B., and Chibbar, R.N. (2007) Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat. *Funct. Integr. Genomics*, **7**, 53–68.

32 Chen, A., Gusta, L.V., Brule-Babel, A., Leach, R., Baumann, U., Fincher, G.B., and Collins, N.C. (2009) Varietal and chromosome 2H locus-specific frost tolerance in reproductive tissues of barley (*Hordeum vulgare* L.) detected using a frost simulation chamber. *Theor. Appl. Genet.*, **119**, 685–694.

33 Dumont, E., Fontaine, V., Vuylsteker, C., Sellier, H., Bodele, S., Voedts, N., Devaux, R., Frise, M., Avia, K., Hilbert, J.-L., Bahrmann, N., Hanocq, E., Lejeune-Henaut, I., and Delbreil, B. (2009) Association of sugar content QTL and PQL with physiological traits relevant to frost damage resistance in pea under field and controlled conditions. *Theor. Appl. Genet.*, **118**, 1561–1571.

34 Gery, C., Zuther, E., Schulz, E., Legoupi, J., Chauveau, A., McKhann, H.I., Hincha, D.K., and Teoule, E. (2011) Natural variation in the freezing tolerance of *Arabidopsis thaliana*: effects of RNAi-induced CBF depletion and QTL localisation vary among accessions. *Plant Sci.*, **180**, 12–23.

35 Wooten, D.R., Livingston, D.P., Holland, J.B., Marshall, D.S., and Murphy, J.P. (2008) Quantitative trait loci and epistasis for crown freezing tolerance in the "Kanota" × "Ogle" hexaploid oat mapping population. *Crop Sci.*, **48**, 149–157.

36 Wooten, D.R., Livingston, D.P., Lyerly, H.J., Holland, J.B., Jellen, E.N., Marshall, D.S., and Murphy, J.P. (2009) Quantitative trait loci and epistasis for oat winter-hardiness component traits. *Crop Sci.*, **49**, 1989–1998.

37 Xiong, Y., Fei, S., Arora, R., Brummer, E.C., Barker, R.E., Jung, G., and Warnke, S.E. (2007) Identification of quantitative trait loci controlling winter hardiness in an annual × perennial ryegrass interspecific hybrid population. *Mol. Breed.*, **19**, 125–136.

38 McKhann, H.I., Gery, C., Berard, A., Leveque, S., Zuther, E., Hincha, D.K., de Mita, S., Brunel, D., and Teoule, E. (2008) Natural variation in CBF gene sequence, gene expression and freezing tolerance in the Versailles core collection of *Arabidopsis thaliana*. *BMC Plant Biol.*, **8**, 105.

39 Rohde, P., Hincha, D.K., and Heyer, A.G. (2004) Heterosis in the freezing tolerance of crosses between two *Arabidopsis thaliana* accessions (Columbia-0 and C24) that show differences in non-acclimated and acclimated freezing tolerance. *Plant J.*, **38**, 790–799.

40 Zhen, Y. and Ungerer, M.C. (2008) Clinal variation in freezing tolerance among natural accessions of *Arabidopsis thaliana*. *New Phytol.*, **177**, 419–427.

41 de Meaux, J. and Koornneef, M. (2008) The cause and consequences of natural variation: the genomic era takes off! *Curr. Opin. Plant Biol.*, **11**, 99–102.

42 Gur, A. and Zamir, D. (2004) Unused natural variation can lift yield barriers in plant breeding. *PLoS Biol.*, **2**, e245.

43 Smallwood, M. and Bowles, D.J. (2002) Plants in a cold climate. *Phil. Trans. R. Soc. Lond. B*, **357**, 831–847.

44 Chinnusamy, V., Zhu, J., and Zhu, J.-K. (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci.*, **12**, 444–451.

45 Knight, H. and Knight, M.R. (2001) Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci.*, **6**, 262–267.

46 Thomashow, M.F. (2010) Molecular basis of plant cold acclimation: insights

gained from studying the CBF cold response pathway. *Plant Physiol.*, **154**, 571–577.

47 van Buskirk, H.A. and Thomashow, M.F. (2006) *Arabidopsis* transcription factors regulating cold acclimation. *Physiol. Plant.*, **126**, 72–80.

48 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2005) Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci.*, **10**, 88–94.

49 Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M., and Thomashow, M.F. (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *Plant J.*, **16**, 433–442.

50 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*, **10**, 1391–1406.

51 Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA*, **94**, 1035–1040.

52 Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994) The 5′-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol. Biol.*, **24**, 701–713.

53 Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell*, **6**, 251–264.

54 Hannah, M.A., Heyer, A.G., and Hincha, D.K. (2005) A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. *PLoS Genet.*, **1**, e26.

55 Gilmour, S.J., Fowler, S.G., and Thomashow, M.F. (2004) *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol. Biol.*, **54**, 767–781.

56 Jaglo-Ottosen, K., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science*, **280**, 104–106.

57 Cook, D., Fowler, S., Fiehn, O., and Thomashow, M.F. (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **101**, 15243–15248.

58 Fowler, S. and Thomashow, M.F. (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell*, **14**, 1675–1690.

59 Maruyama, K., Takeda, M., Kidokoro, S., Yamada, K., Sakuma, Y., Urano, S., Fujita, M., Yoshiwara, K., Matsukura, S., Morishita, Y., Sasaki, R., Suzuki, H., Saito, K., Shibata, D., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2009) Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. *Plant Physiol.*, **150**, 1972–1980.

60 Novillo, F., Alonso, J.M., Ecker, J.R., and Salinas, J. (2004) CBF2/DREB1C is a negative regulator of *CBF1/DREB1B* and *CBF3/DREB1A* expression and plays a central role in stress tolerance in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **101**, 3985–3990.

61 Novillo, F., Medina, J., and Salinas, J. (2007) *Arabidopsis* CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. *Proc. Natl. Acad. Sci. USA*, **104**, 21002–21007.

62 Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B., Hong, X., Agarwal, M., and Zhu, J.-K. (2003) ICE1: a regulator of

cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.*, **17**, 1043–1054.

63 Kanaoka, M., Pillitteri, L.J., Fuji, H., Yoshida, Y., Bogenschutz, N.L., Takabayashi, J., Zhu, J.-K., and Torii, K.U. (2008) *SCREAM/ICE1* and *SCREAM2* specify three cell-state transitional steps leading to *Arabidopsis* stomatal differentiation. *Plant Cell*, **20**, 1775–1785.

64 Lee, B., Henderson, D.A., and Zhu, J.-K. (2005) The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell*, **17**, 3155–3175.

65 Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B., and Zhu, J.-K. (2001) The *Arabidopsis HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. *Genes Deve.*, **15**, 912–924.

66 Dong, C.H., Agarwal, M., Zhang, Y., Xie, Q., and Zhu, J.-K. (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proc. Natl. Acad. Sci. USA*, **103**, 8281–8286.

67 Knight, H., Mugford, S.G., Ulker, B., Gao, D., Thorlby, G., and Knight, M.R. (2009) Identification of SFR6, a key component in cold acclimation acting post-translationally on CBF function. *Plant J.*, **58**, 97–108.

68 Guo, Y., Xiong, L., Ishitani, M., and Zhu, J.-K. (2002) An *Arabidopsis* mutation in translation elongation factor 2 causes superinduction of *CBF/DREB1* transcription factor genes but blocks the induction of their downstream targets under low temperatures. *Proc. Natl. Acad. Sci. USA*, **99**, 7786–7791.

69 Gong, Z., Lee, H., Xiong, L., Jagendorf, A., Stevenson, B., and Zhu, J.-K. (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc. Natl. Acad. Sci. USA*, **99**, 11507–11512.

70 Vogel, J.T., Zarka, D.G., van Buskirk, H.A., Fowler, S.G., and Thomashow, M.F. (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *Plant J.*, **41**, 195–211.

71 Achard, P., Cheng, H., de Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., van der Straeten, D., Peng, J., and Harberd, N.P. (2006) Integration of plant responses to environmentally activated phytohormone signals. *Science*, **311**, 91–94.

72 Achard, P., Gong, F., Cheminant, S., Alioua, M., Hedden, P., and Genschik, P. (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberrellin metabolism. *Plant Cell*, **20**, 2117–2129.

73 Xin, Z., Mandaokar, A., Chen, J., Last, R.L., and Browse, J. (2007) *Arabidopsis ESK1* encodes a novel regulator of freezing tolerance. *Plant J.*, **49**, 786–799.

74 Haake, V., Cooke, D., Riechmann, J.L., Pineda, O., Thomashow, M.F., and Zhang, J.Z. (2002) Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. *Plant Physiol.*, **130**, 639–648.

75 Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T., and Thomashow, M.F. (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.*, **127**, 910–917.

76 Zhang, X., Fowler, S.G., Cheng, H., Lou, Y., Rhee, S.Y., Stockinger, E.J., and Thomashow, M.F. (2004) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. *Plant J.*, **39**, 905–919.

77 Skinner, J.S., von Zitzewitz, J., Szucs, P., Marquez-Cedillo, L., Filichkin, T., Amundsen, K., Stockinger, E.J., Thomashow, M.F., Chen, T.H.H., and Hayes, P.M. (2005) Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley. *Plant Mol. Biol.*, **59**, 533–551.

78 Stockinger, E.J., Skinner, J.S., Gardner, K.G., Francia, E., and Pecchioni, N. (2007) Expression levels of barley *Cbf* genes at the *Frost resistance-H2* locus are dependent upon alleles at *FrH1* and *Fr-H2*. *Plant J.*, **51**, 308–321.

79 Benedict, C., Skinner, J.S., Meng, R., Chang, Y., Bhalerao, R., Huner, N.P.A., Finn, C.E., Chen, T.H.H., and Hurry, V. (2006) The CBF1-dependent low temperature signalling pathway, regulon and increase in freezing tolerance are conserved in *Populus* spp. *Plant Cell Environ.*, **29**, 1259–1272.

80 El Kayal, W., Navarro, M., Marque, G., Keller, G., Marque, C., and Teulieres, C. (2006) Expression profile of *CBF*-like transcriptional factor genes from *Eucalyptus* in response to cold. *J. Exp. Bot.*, **57**, 2455–2469.

81 Somero, G.N. (1992) Adapting to water stress: convergence on common solutions, in *Water and Life* (eds G.N. Somero, C.B. Osmond, and C.L. Bolis), Springer, Berlin, pp. 3–18.

82 Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D., and Somero, G.N. (1982) Living with water stress: evolution of osmolyte systems. *Science*, **217**, 1214–1222.

83 Timasheff, S.N. (1993) The control of protein stability and association by weak interactions with water: how do solvents affect these processes? *Annu. Rev. Biophys. Biomol. Struct.*, **22**, 67–97.

84 Diamant, S., Eliahu, N., Rosenthal, D., and Goloubinoff, P. (2001) Chemical chaperones regulate molecular chaperones *in vitro* and cells under combined salt and heat stresses. *J. Biol. Chem.*, **276**, 39586–39591.

85 Crowe, J.H., Carpenter, J.F., Crowe, L.M., and Anchordoguy, T.J. (1990) Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. *Cryobiology*, **27**, 219–231.

86 Hincha, D.K., Popova, A.V., and Cacela, C. (2006) Effects of sugars on the stability of lipid membranes during drying, in *Advances in Planar Lipid Bilayers and Liposomes* (ed. A. Leitmannova Liu), Elsevier, Amsterdam, pp. 189–217.

87 Hincha, D.K., Zuther, E., Hundertmark, M., and Heyer, A.G. (2005) The role of compatible solutes in plant freezing tolerance: a case study on raffinose, in *Cold Hardiness in Plants: Molecular Genetics, Cell Biology and Physiology* (eds T.H.H. Chen, M. Uemura, and S. Fujikawa), CABI Publishing, Wallingford, UK, pp. 203–218.

88 Livingston, D.P., Hincha, D.K., and Heyer, A.G. (2009) Fructan and its relationship to abiotic stress tolerance in plants. *Cell Mol. Life Sci.*, **66**, 2007–2023.

89 Verbruggen, N. and Hermans, C. (2008) Proline accumulation in plants: a review. *Amino Acids*, **35**, 753–759.

90 Chen, T.H.H. and Murata, N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.*, **5**, 250–257.

91 Nuccio, M.L., Rhodes, D., McNeil, S.D., and Hanson, A.D. (1999) Metabolic engineering of plants for osmotic stress resistance. *Curr. Opin. Plant Biol.*, **2**, 128–134.

92 Kandpal, R. and Rao, N. (1985) Alterations in the biosynthesis of proteins and nucleic acids in finger millet (*Eleucine coracana*) seedlings during water stress and the effect of proline on protein biosynthesis. *Plant Sci*, **40**, 73–79.

93 Nikolopoulos, D. and Manetas, Y. (1991) Compatible solutes and *in vitro* stability of *Salsola soda* enzymes: proline incompatibility. *Phytochemistry*, **30**, 411–413.

94 Harvaux, M. and Kloppstech, K. (2001) The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in *Arabidopsis* npq and tt mutants. *Planta*, **213**, 953–966.

95 Nishizawa, A., Yabuta, Y., and Shigeoka, S. (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol.*, **147**, 1251–1263.

96 Delseny, M., Han, B., and Hsing, Y. (2010) High throughput DNA sequencing: the new sequencing revolution. *Plant Sci.*, **179**, 407–422.

97 Rodriguez, M.C.S., Edsgard, D., Hussain, S.S., Alquezar, D., Rasmussen, M., Gilbert, T., Nielsen, B.H., Bartels, D., and Mundy, J. (2010) Transcriptomes of the desiccation-tolerant resurrection plant *Craterostigma plantagineum*. *Plant J.*, **63**, 212–228.

98 Amme, S., Matros, A., Schlesier, B., and Mock, H.-P. (2006) Proteome analysis of cold stress response in *Arabidopsis thaliana* using DIGE-technology. *J. Exp. Bot.*, **57**, 1537–1546.

99 Gao, F., Zhou, Y., Zhu, W., Li, X., Fan, L., and Zhang, G. (2009) Proteomic analysis of cold stress-responsive proteins in *Thellungiella* rosette leaves. *Planta*, **230**, 1033–1046.

100 Goulas, E., Schubert, M., Kieselbach, T., Kleczkowski, L.A., Gardeström, P., Schröder, W., and Hurry, V. (2006) The chloroplast lumen and stromal proteomes of *Arabidopsis thaliana* show differential sensitivity to short- and long-term exposure to low temperature. *Plant J.*, **47**, 720–734.

101 Kamal, A.H.M., Kim, K.-H., Shin, K.-H., Choi, J.-S., Baik, B.-K., Tsujimoto, H., Heo, H.Y., Park, C.-S., and Woo, S.-H. (2010) Abiotic stress responsive proteins of wheat grain determined using proteomics technique. *Austr. J. Crop Sci.*, **4**, 196–208.

102 Kawamura, Y. and Uemura, M. (2003) Mass spectrometric approach to identifying putative plasma membrane proteins of *Arabidopsis* leaves associated with cold acclimation. *Plant J.*, **36**, 141–154.

103 Kjellsen, T.D., Shiryaeva, L., Schröder, W., and Strimbeck, G.R. (2010) Proteomics of extreme freezing tolerance in Siberian spruce (*Picea obovata*). *J. Proteom.*, **73**, 965–975.

104 Renaut, J., Lutts, S., Hoffmann, L., and Hausmann, J.-F. (2004) Responses of poplar to chilling temperatures: proteomic and physiological aspects. *Plant Biol.*, **6**, 81–90.

105 Korn, M., Peterek, S., Mock, H.-P., Heyer, A.G., and Hincha, D.K. (2008) Heterosis in the freezing tolerance, and sugar and flavonoid contents of crosses between *Arabidopsis thaliana* accessions of widely varying freezing tolerance. *Plant Cell Environ.*, **31**, 813–827.

106 Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., and Harter, K. (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J.*, **50**, 347–363.

107 Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., and Harper, J.F. (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol.*, **130**, 2129–2141.

108 Oono, Y., Seki, M., Satou, M., Iida, K., Akiyama, K., Sakurai, T., Fujita, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Monitoring expression profiles of *Arabidopsis* genes during cold acclimation and deacclimation using DNA microarrays. *Funct. Integr. Genomics*, **6**, 212–234.

109 Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., and Shinozaki, K. (2002) Monitoring of the expression profiles of 7000 *Arabidopsis* genes under drought, cold, and high-salinity stresses using a full-length cDNA microarray. *Plant J.*, **31**, 279–292.

110 Soitamo, A.J., Piippo, M., Allahverdiyeva, Y., Battchikova, N., and Aro, E.M. (2008) Light has a specific role in modulating *Arabidopsis* gene expression at low temperature. *BMC Plant Biol.*, **8**, 13.

111 Maruyama, K., Sakuma, Y., Kasuga, M., Ito, Y., Seki, M., Goda, H., Shimada, Y., Yoshida, S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004) Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. *Plant J.*, **38**, 982–993.

112 Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. (2001) Monitoring the

expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell*, **13**, 61–72.

113 Kaplan, F., Kopka, J., Sung, D.Y., Zhao, W., Popp, M., Porat, R., and Guy, C.L. (2007) Transcript and metabolite profiling during cold acclimation of *Arabidopsis* reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. *Plant J.*, **50**, 967–981.

114 Usadel, B., Blasing, O.E., Gibon, Y., Poree, F., Hohne, M., Gunter, M., Trethewey, R., Kamlage, B., Poorter, H., and Stitt, M. (2008) Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in *Arabidopsis* rosettes to a progressive decrease of temperature in the non-freezing range. *Plant Cell Environ.*, **31**, 518–547.

115 Espinoza, C., Degenkolbe, T., Caldana, C., Zuther, E., Leisse, A., Willmitzer, L., Hincha, D.K., and Hannah, M.A. (2010) Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in *Arabidopsis*. *PLoS ONE*, **5**, e14101.

116 Gulick, P.J., Drouin, S., Yu, Z., Danyluk, J., Poisson, G., Monroy, A.F., and Sarhan, F. (2005) Transcriptome comparison of winter and spring wheat responding to low temperature. *Genome*, **48**, 913–923.

117 Kocsy, G., Athmer, B., Perovic, D., Himmelbach, A., Szucs, A., Vashegyi, I., Schweizer, P., Galiba, G., and Stein, N. (2010) Regulation of gene expression by chromosome 5A during cold hardening in wheat. *Mol. Genet. Genom.*, **283**, 351–363.

118 Monroy, A.F., Dryanova, A., Malette, B., Oren, D.H., Ridha Farajalla, M., Liu, W., Danyluk, J., Ubayasena, L.W., Kane, K., Scoles, G.J., Sarhan, F., and Gulick, P.J. (2007) Regulatory gene candidates and gene expression analysis of cold acclimation in winter and spring wheat. *Plant Mol. Biol.*, **64**, 409–423.

119 Svensson, J.T., Crosatti, C., Campoli, C., Bassi, R., Stanca, A.M., Close, T.J., and Cattivelli, L. (2006) Transcriptome analysis of cold acclimation in barley *albina* and *xantha* mutants. *Plant Physiol.*, **141**, 257–270.

120 Tommasini, L., Svensson, J.T., Rodriguez, E.M., Wahid, A., Malatrasi, M., Kato, K., Wanamaker, S., Resnik, J., and Close, T.J. (2008) Dehydrin gene expression provides an indicator of low temperature and drought stress: transcriptome-based analysis of barley (*Hordeum vulgare* L.). *Funct. Integr. Genomics*, **8**, 387–405.

121 Dhanaraj, A.L., Alkharouf, N.W., Beard, H.S., Chouikha, I.B., Matthews, B.F., Wei, H., Arora, R., and Rowland, L.J. (2007) Major differences observed in transcript profiles of blueberry during cold acclimation under field and cold room conditions. *Planta*, **225**, 735–751.

122 Schrader, J., Moyle, R., Bhalerao, R., Hertzberg, M., Lundeberg, J., Nilsson, P., and Bhalerao, R.P. (2004) Cambial meristem dormancy in trees involves extensive remodelling of the transcriptome. *Plant J.*, **40**, 173–187.

123 Joosen, R.V., Lammers, M., Balk, P.A., Konings, M.C., Perks, M., Stattin, E., van Wordragen, M.F., and van der Geest, A.L. (2006) Correlating gene expression to physiological parameters and environmental conditions during cold acclimation of *Pinus sylvestris*, identification of molecular markers using cDNA microarrays. *Tree Physiol.*, **26**, 1297–1313.

124 Holliday, J.A., Ralph, S.G., White, R., Bohlmann, J., and Aitken, S.N. (2008) Global monitoring of autumn gene expression within and among phenotypically divergent populations of Sitka spruce (*Picea sitchensis*). *New Phytol.*, **178**, 103–122.

125 Seki, M., Kamei, A., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Curr. Opin. Biotechnol.*, **14**, 194–199.

126 Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M. (2003) Regulatory network of gene expression in the drought and cold

stress responses. *Curr. Opin. Plant Biol.*, **6**, 410–417.

127 Nakashima, K. and Yamaguchi-Shinozaki, K. (2006) Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants. *Physiol. Plant.*, **126**, 62–71.

128 Yang, T., Zhang, L., Zhang, T., Zhang, H., Xu, S., and An, L. (2005) Transcriptional regulation network of cold-responsive genes in higher plants. *Plant Sci.*, **169**, 987–995.

129 Hundertmark, M. and Hincha, D.K. (2008) LEA (Late Embryogenesis Abundant) proteins and their encoding genes in *Arabidopsis thaliana*. *BMC Genomics*, **9**, 118.

130 Garay-Arroyo, A., Colmenero-Flores, J.M., Garciarrubio, A., and Covarrubias, A.A. (2000) Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. *J. Biol. Chem.*, **275**, 5668–5674.

131 Guy, C.L., Kaplan, F., Kopka, J., Selbig, J., and Hincha, D.K. (2008) Metabolomics of temperature stress. *Physiol. Plant.*, **132**, 220–235.

132 Rice-Evans, C.A., Miller, N.J., and Paganga, G. (1997) Antioxidant properties of phenolic compounds. *Trends Plant Sci.*, **2**, 152–159.

133 Winkel-Shirley, B. (2002) Biosynthesis of flavonoids and effects of stress. *Curr. Opin. Plant Biol.*, **5**, 218–223.

134 Wanner, L.A. and Junttila, O. (1999) Cold-induced freezing tolerance in *Arabidopsis*. *Plant Physiol.*, **120**, 391–399.

135 Hugly, S. and Somerville, C. (1992) A role for membrane lipid polyunsaturation in chloroplast biogenesis at low temperature. *Plant Physiol.*, **99**, 197–202.

136 Welling, A. and Palva, E.T. (2006) Molecular control of cold acclimation in trees. *Physiol. Plant.*, **127**, 167–181.

137 Guy, C.L. and Haskell, D. (1987) Induction of freezing tolerance in spinach is associated with the synthesis of cold acclimation induced proteins. *Plant Physiol.*, **84**, 872–878.

138 Lang, V., Mäntylä, E., Welin, B., Sundberg, B., and Palva, E.T. (1994) Alterations in water status, endogenous abscisic acid content, and expression of *rab18* gene during the development of freezing tolerance in *Arabidopsis thaliana*. *Plant Physiol.*, **104**, 1341–1349.

139 Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. (1995) Serial analysis of gene-expression. *Science*, **270**, 484–487.

140 Byun, Y.J., Kim, H.J., and Lee, D.H. (2009) LongSAGE analysis of the early response to cold stress in *Arabidopsis* leaf. *Planta*, **229**, 1181–1200.

141 Jung, S.H., Lee, J.Y., and Lee, D.H. (2003) Use of SAGE technology to reveal changes in gene expression in *Arabidopsis* leaves undergoing cold stress. *Plant Mol. Biol.*, **52**, 553–567.

142 Lee, J.Y. and Lee, D.H. (2003) Use of serial analysis of gene expression technology to reveal changes in gene expression in *Arabidopsis* pollen undergoing cold stress. *Plant Physiol.*, **132**, 517–529.

143 Robinson, S.J. and Parkin, I.A. (2008) Differential SAGE analysis in *Arabidopsis* uncovers increased transcriptome complexity in response to low temperature. *BMC Genomics*, **9**, 434.

144 Saha, S., Sparks, A.B., Rago, C., Akmaev, V., Wang, C.J., Vogelstein, B., Kinzler, K.W., and Velculescu, V.E. (2002) Using the transcriptome to annotate the genome. *Nat. Biotechnol.*, **20**, 508–512.

145 Bieniawska, Z., Espinoza, C., Schlereth, A., Sulpice, R., Hincha, D.K., and Hannah, M.A. (2008) Disruption of the *Arabidopsis* circadian clock is responsible for extensive variation in the cold-responsive transcriptome. *Plant. Physiol.*, **147**, 263–279.

146 Ibanez, C., Ramos, A., Acebo, P., Contreras, A., Casado, R., Allona, I., and Aragoncillo, C. (2008) Overall alteration of circadian clock gene expression in the chestnut cold response. *PLoS ONE*, **3**, e3567.

147 Ramos, A., Perez-Solis, E., Ibanez, C., Casado, R., Collada, C., Gomez, L., Aragoncillo, C., and Allona, I. (2005) Winter disruption of the circadian clock in chestnut. *Proc. Natl. Acad. Sci. USA*, **102**, 7037–7042.

148 Czechowski, T., Bari, R., Stitt, M., Scheible, W.-R., and Udvardi, M. (2004) Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors:

unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant J.*, **38**, 366–379.

149 Fowler, S.G., Cook, D., and Thomashow, M.F. (2005) Low temperature induction of *Arabidopsis CBF1, 2,* and *3* is gated by the circadian clock. *Plant Physiol.*, **137**, 961–968.

150 Wilkins, O., Bräutigam, K., and Campbell, M.M. (2010) Time of day shapes *Arabidopsis* drought transcriptomes. *Plant J.*, **63**, 715–727.

151 Swindell, W.R., Huebner, M., and Weber, A.P. (2007) Plastic and adaptive gene expression patterns associated with temperature stress in *Arabidopsis thaliana*. *Heredity*, **99**, 143–150.

152 Kaplan, F., Kopka, J., Haskell, D.W., Zhao, W., Schiller, K.C., Gatzke, N., Sung, D.Y., and Guy, C.L. (2004) Exploring the temperature-stress metabolome of *Arabidopsis*. *Plant Physiol.*, **136**, 4159–4168.

153 Gray, G.R. and Heath, D. (2005) A global reorganization of the metabolome in *Arabidopsis* during cold acclimation is revealed by metabolic fingerprinting. *Physiol. Plant.*, **124**, 236–248.

154 Bläsing, O.E., Gibon, Y., Günther, M., Höhne, M., Morcuende, R., Osuna, D., Thimm, O., Usadel, B., Scheible, W.-R., and Stitt, M. (2005) Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in *Arabidopsis*. *Plant Cell*, **17**, 3257–3281.

155 Zeeman, S.C., Smith, S.M., and Smith, A.M. (2007) The diurnal metabolism of starch. *Biochem. J.*, **401**, 11–28.

156 Stitt, M. and Hurry, V. (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. *Curr. Opin. Plant Biol.*, **5**, 199–206.

157 Hurry, V.M., Keerberg, O., Pärnik, T., Gardeström, P., and Öquist, G. (1995) Cold-hardening results in increased activity of enzymes involved in carbon metabolism of winter rye (*Secale cereale* L.). *Planta*, **195**, 554–562.

158 Strand, A., Hurry, V., Gustafsson, P., and Gardeström, P. (1997) Development of *Arabidopsis thaliana* leaves at low temperatures releases the suppression of photosynthesis and photosynthetic gene expression despite the accumulation of soluble carbohydrates. *Plant J.*, **12**, 605–614.

159 Zuther, E., Büchel, K., Hundertmark, M., Stitt, M., Hincha, D.K., and Heyer, A.G. (2004) The role of raffinose in the cold acclimation response of *Arabidopsis thaliana*. *FEBS Lett.*, **576**, 169–173.

160 Korn, M., Gärtner, T., Erban, A., Kopka, J., Selbig, J., and Hincha, D.K. (2010) Predicting *Arabidopsis* freezing tolerance and heterosis in freezing tolerance from metabolite composition. *Mol. Plant*, **3**, 224–235.

161 Kaplan, F. and Guy, C.L. (2005) RNA interference of *Arabidopsis* beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. *Plant J.*, **44**, 730–743.

162 Hincha, D.K., Zuther, E., Hellwege, E.M., and Heyer, A.G. (2002) Specific effects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying. *Glycobiology*, **12**, 103–110.

163 Kaplan, F. and Guy, C.L. (2004) β-Amylase induction and the protective role of maltose during temperature shock. *Plant Physiol.*, **135**, 1674–1684.

164 Yano, R., Nakamura, M., Yoneyama, T., and Nishida, I. (2005) Starch-related alpha-glucan/water dikinase is involved in the cold-induced development of freezing tolerance in *Arabidopsis*. *Plant Physiol.*, **138**, 837–846.

165 Dixon, R.A. and Pasinetti, G.M. (2010) Flavonoids and isoflavonoids: from plant biology to agriculture and neuroscience. *Plant Physiol.*, **154**, 453–457.

166 Janska, A., Marsik, P., Zelenkova, S., and Ovesna, J. (2010) Cold stress and acclimation – what is important for metabolic adjustment? *Plant Biol.*, **12**, 395–405.

167 Winkel-Shirley, B. (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.*, **126**, 485–493.

168 Hernandez, I. and van Breusegem, F. (2010) Opinion on the possible role of flavonoids as energy escape valves: novel tools for nature's Swiss army knife. *Plant Sci.*, **179**, 297–301.

169 Krol, M., Gray, G.R., Hurry, V.M., Oquist, G., Malek, L., and Huner, N.P.A. (1995) Low-temperature stress and photoperiod affect an increased tolerance to photoinhibition in *Pinus banksiana* seedlings. *Can. J. Bot.*, **73**, 1119–1127.

170 Scheidt, H.A., Pampel, A., Nissler, L., Gebhardt, R., and Huster, D. (2004) Investigation of the membrane localization and distribution of flavonoids by high-resolution magic angle spinning NMR spectroscopy. *Biochim. Biophys. Acta*, **1663**, 97–107.

171 Hoekstra, F.A. and Golovina, E.A. (2002) The role of amphiphiles. *Comp. Biochem. Physiol.*, **131**, 527–533.

172 Hincha, D.K., Oliver, A.E., and Crowe, J.H. (1999) Lipid composition determines the effects of arbutin on the stability of membranes. *Biophys. J.*, **77**, 2024–2034.

173 Oliver, A.E., Leprince, O., Wolkers, W.F., Hincha, D.K., Heyer, A.G., and Crowe, J.H. (2001) Non-disaccharide-based mechanisms of protection during drying. *Cryobiology*, **43**, 151–167.

174 Quinn, P.J. (1985) A lipid-phase separation model of low-temperature damage to biological membranes. *Cryobiology*, **22**, 128–146.

175 Nishida, I. and Murata, N. (1996) Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 541–568.

176 Iba, K. (2002) Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. *Annu. Rev. Plant Biol.*, **53**, 225–245.

177 Murata, N., Ishizaki-Nishizawa, S., Higashi, S., Hayashi, H., Tasaka, Y., and Nishida, I. (1992) Genetically engineered alteration in the chilling sensitivity of plants. *Nature*, **356**, 710–713.

178 Wolter, F., Schmidt, R., and Heinz, E. (1992) Chilling sensitivity of *Arabidopsis thaliana* with genetically engineered membrane lipids. *EMBO J.*, **11**, 4685–4692.

179 Miquel, M., James, D., Dooner, H., and Browse, J. (1993) *Arabidopsis* requires polyunsaturated lipids for low-temperature survival. *Proc. Natl. Acad. Sci. USA*, **90**, 6208–6212.

180 Wu, J., Lightner, J., Warwick, N., and Browse, J. (1997) Low-temperature damage and subsequent recovery of *fab1* mutant *Arabidopsis* exposed to 2 °C. *Plant Physiol.*, **113**, 347–356.

181 Moellering, E.R., Muthan, B., and Benning, C. (2010) Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. *Science*, **330**, 226–228.

182 Uemura, M. and Steponkus, P.L. (1999) Cold acclimation in plants: relationship between the lipid composition and the cryostability of the plasma membrane. *J. Plant Res.*, **112**, 245–254.

183 Lewandowska, M. and Sirko, A. (2008) Recent advances in understanding plant response to sulfur-deficiency stress. *Acta Biochim. Pol.*, **55**, 457–471.

184 Zheng, Z.L. (2009) Carbon and nitrogen nutrient balance signaling in plants. *Plant Signal. Behav.*, **4**, 584–591.

185 Bouche, N. and Fromm, H. (2004) GABA in plants: just a metabolite? *Trends Plant Sci.*, **9**, 110–115.

186 Fait, A., Fromm, H., Walter, D., Galili, G., and Fernie, A.R. (2008) Highway or byway: the metabolic role of the GABA shunt in plants. *Trends Plant Sci.*, **13**, 14–19.

187 Mazzucotelli, E., Tartari, A., Cattivelli, L., and Forlani, G. (2006) Metabolism of gamma-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat. *J. Exp. Bot.*, **57**, 3755–3766.

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Omics Techniques in Crop Research: An Overview

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12.1
Introduction

Omics is a collective, broad discipline largely referring to analysis of the interactions of biological information obtained from the profiling of the genome, transcriptome, proteome, metabolome, and several other relevant -omes. While phase one of omics technologies aims at nontargeted identification of transcripts, proteins, and metabolites (essentially gene products) in a given biological sample, phase two deals with a very challenging analysis of data eventually leading to the dissection of the qualitative and quantitative dynamics of biological systems. Essentially, the omics science is enabled by a host of diverse, high-throughput technologies and platforms [1]. The full range of omics technologies can now be applied to understand the same fundamental biological processes [2]. Mapping and defining the relationships among genes, proteins, and metabolites require relative comparison of the networks that eventually help in understanding the regulatory mechanisms. A diverse but converging approaches such as forward and reverse genetics and transgenics (overexpression and knockdowns) can define the function of a gene to the specific phenotype, the omic technologies aim at revealing the function of each and every gene in the genome, which collectively contribute toward elucidating the networks and better understanding the whole plant phenotype [3–5]. Access to omics tools at an affordable price is becoming a reality, which together with a large inventory of candidate genes, proteins, and metabolites and their databases deduced from profiling efforts in model systems and crop plants have speeded up the analysis of biological functions operating in various plant stress responses [2, 6]. These new strategies have begun to piece together the physiological and phenotypic observations with information on transcription and transcript regulation, the behavior of proteins, protein complexes and pathways, and the metabolites and metabolite fluxes, finally shedding light on evolutionary adaptive diversifications of organisms.

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12.2 Transcriptomics

Transcriptomics, a global mRNA expression profiling of a particular tissue, is essentially genome wide, yielding information about the transcriptional differences between defined states of tissues. Elucidated global differences in gene expression are expected to help in the understanding of genes and pathways involved in biological processes: gene statements showing similarity in quantitative and qualitative expression are functionally related and would be the result of possible common genetic regulation [2, 7]. Rapid sequencing of many eukaryotic genomes has provided unprecedented opportunities to understand gene function, genome structure, and genome evolution [8]. However, an accurate annotation of all expressed genes in the sequenced genomes remains one of the most challenging tasks. Therefore, genomic resources and platforms provide new opportunities for crop research and breeding programs [8–10].

Transcriptomics can be used to understand taxonomic position to gain a deeper understanding of molecular and physiological bases of complex phenotypes such as crop response to abiotic stresses. Common platform technologies used for genome-wide or high-throughput analysis of gene expression are microarrays, serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), and next-generation sequencing platforms (NGSPs) [11–13]. High-throughput quantitative real-time PCR has proven to be a platform of choice for validation of a large number of genes elucidated through omics approaches in the tissue and treatment of choice [14].

12.2.1 Closed Omics Technologies

Microarray technology represents a "closed" profiling strategy limited by the target genes/gene statements imprinted on gene chips. Microarray technology has become a useful tool for the analysis of genome-scale gene expression. This technology was first demonstrated by analyzing 48 *Arabidopsis* genes for differential expression in roots and shoots [15]. Microarrays are artificially constructed grids of known DNA samples such that each element of the grid probes a specific RNA sequence wherein the RNA transcripts from the target sample are captured and quantified. To date, many different protocols and types of microarrays such as oligonucleotide and cDNA arrays, commercially available whole-genome arrays and custom-made, tissue-specific arrays have been developed. All these invariably require (a) isolation of RNA from target sample, (b) conversion of RNA to either cDNA or cRNA, (c) a simultaneous incorporation of either fluorescent nucleotides or a tag that is later used for fluorescent labeling, (d) hybridization to a chosen microchip, (e) washing and labeling (depending on the protocol adopted), (f) scanning under laser light and image processing to extract data, and (g) data analysis [16, 17]. Bioinformatic clustering tools are required for delineation of closely related expression patterns of genes [17]. The sources for cDNA arrays are generally PCR products from expressed sequence tags (ESTs) or from cell (tissue)-specific sources, which are "spotted" on glass slides. In the case of oligonucleotide

arrays, relatively short, 25-mer, oligonucleotides specific for transcripts of interest are generated by photolithography and solid-phase DNA synthesis [18]. Various modifications to these approaches and the use of fluorophore dyes have allowed a more accurate and reliable expression profiling.

Microarrays have been extensively used in most of the experimental systems including major crop plants [19–22]. For instance, in *Arabidopsis*, full-length cDNA libraries from plants under different conditions, such as drought-treated and cold-treated plants, have been developed [23, 24] and a set of 1300 full-length cDNAs were monitored for the expression patterns under drought and cold stresses [25]. This platform has been widely used in crop research for elucidation of differentially expressed genes in crop plants as a result of biotic and abiotic stress interactions [7, 21, 26].

There are two microarray-based methods for genotyping in crop research: (a) one involves arraying thousands of short oligonucleotides on glass slides for detection of many single-nucleotide polymorphism (SNP) loci in target DNA, which is particularly well suited for genotyping thousands of markers, and the other (b) involves arraying amplified PCR products on glass slides to detect a few SNPs. The latter is useful to detect limited number of SNPs in large number of samples [27, 28]. Biotin-terminated, allele-specific PCR products are spotted unpurified on glass slides coated with streptavidin and visualized through fluorescent oligonucleotides attached to the allele-specific PCR primers. These approaches of genotyping hold great promise in high-throughput genotyping the candidate genes and their trait association in crop plants. A maize expression array containing 57 452 genes has been developed and used in the context of maize nitrogen utilization, root growth under drought, water, and phosphate stress, seed development, photosynthesis, pathogen response, aluminum stress in roots, tassel development, and hybrid vigor (www.maizearray.org). Furthermore, microarray-based gene expression technology is a powerful tool to also monitor changes in the expression of a large number of genes simultaneously and provide new insights into physiological and biochemical pathways of abiotic and biotic stress tolerance and identify novel candidate genes that can be used in plant breeding programs [6, 17, 29].

12.2.2 Open Omics Technologies

In the past decade, various sequencing-based strategies, such as ESTs [30], full-length cDNA [24], SAGE [31, 32], and MPSS have been developed for transcriptome studies [33, 34]. These approaches have contributed valuable resources for gene discovery and genome annotation, but their application in most molecular studies has been limited. In contrast to microarray technology, these technologies are of "open" architectural systems that can be used to identify novel genes and to quantify differentially expressed mRNAs.

12.2.2.1 ESTs, SAGE, and MPSS

Single-pass sequencing of cDNAs to generate ESTs has been a much-used method of elucidation of genes and has contributed a lot of entries to public DNA databases. The error-prone ESTs have remained not only a powerful means of gene discovery but also

a source of biologically informative probes in genome mapping and cloning studies. ESTs have become an invaluable resource for gene discovery, genome annotation, alternative splicing, SNP discovery, molecular markers for population analysis, and expression analysis in animal, plant, and microbial species [16, 35]. Generally, EST and full-length cDNA sequencing techniques are not deep enough to isolate rare transcripts responsible for complex traits or address transcript variability that persists within and between closely related pathways and phenotypic traits. Sequencing millions of cDNA clones from various tissues can sample only about 60% of the expressed genes [36]. Although various computer-based gene prediction methods play a role in genome annotation, experimental data are an essential evidence for determination of gene structure and function [12]. This limitation has been addressed through high-throughput and short tag-based approaches such as SAGE and MPSS, and much recently employing NGSP. Notably, these technologies are most useful for gene expression analysis in plant species whose genome has been sequenced [11, 37].

The SAGE library construction involves several tedious steps before tags can be cloned into a plasmid vector. The process includes isolation of short tags (14–26 bp) from the 3′ or 5′ ends of transcripts, ditag formation, and concatenation and sequencing of SAGE clones. Taking advantage of the high-throughput made possible by the classical SAGE technology [32], new approaches for cloning of 5′-end-specific sequencing tags from mRNA-cap analysis gene expression (CAGE), *trans*-spliced exon-coupled RNA-end determination (TEC-RED), and 5′ serial analysis of gene expression (5′SAGE), gene identification signatures (GIS), the tags comprising information from both terminal ends, were developed [12, 31]. However, the time-consuming procedure of colony picking and storage and the high cost of sequencing individual clones in SAGE library construction have discouraged the use of this approach in many biological studies [31, 38].

The MPSS strategy involves *in vitro* cloning of cDNA molecules on the surface of microbeads and nongel-based sequencing of millions of tags (17–20 bp). It is considered to be more sensitive over SAGE technology [36, 38]. The multiple location-matching of 17–21 bp tags from SAGE or MPSS libraries in a sequenced genome is problematic when mapping tags to the EST or genomic sequence. To obtain accurate matches for positive tags in the genome, longer transcripts are required. This is usually accomplished using techniques such as rapid amplification of cDNA ends (RACE) or generation of longer cDNA fragments using the GLGI method. Integration of pyrosequencing in sequencing technology with SAGE tags has resulted in an increased sensitivity for deep transcript profiling: robust analysis of 5′ gene expression (5′RATE), which involves the use of pyrosequencing of ditag libraries, achieves higher sensitivity of transcript profiling. It consists of three major steps including 5′-oligocapping of mRNA, *Nla*III tag and ditag generation, and pyrosequencing of *Nla*III tags. Complicated steps such as purification and cloning of concatemers, colony picking, and plasmid DNA purification are eliminated and the conventional Sanger sequencing method is replaced with the newly developed pyrosequencing method [39]. Taken together, these techniques provide a panoramic profile of the entire pool of mRNA transcripts that make up the transcriptome.

12.2.2.2 Next-Generation Sequencing

At present, numerous strategies and platforms are under development including sequencing by synthesis (SBS), sequencing by hybridization, and nanopore sequencing. In 2005, two new sequencing technologies were introduced. Both are based on sequencing by synthesis: the 454 system (http://www.454.com) using pyrosequencing technology and the Solexa/Illumina system (http://www.illumina.com) that depends on detection of fluorescence signals [40]. These methods employ parallel sequencing in millions of reactions that generate a very large number of data points. The read lengths are averaging 100–230 bp and 300–400 bp for 454FLX and 454Titanium, respectively, and 35–105 bp for Illumina Solexa platforms. The Applied Biosystems SOLiD (http://www3.appliedbiosystems.com) is another addition with a greater potential in transcriptome sequencing and gene discovery approaches. These platforms offer a variety of experimental approaches for characterizing a transcriptome, discovering genes, small RNAs, and variations in homologues [8, 11, 41]. These sequencing technologies collectively are referred to as next-generation sequencing (NGS) technologies. Potential applications of NGS technologies in gene expression analysis and crop breeding research have been accounted and compared in detail [8, 9, 13, 42, 43]. In addition, several organizations are working on third-generation sequencing technology mainly based on single-molecule synthesis [44]. Furthermore, both the nanopore sequencing and the transmission electron microscopy-based sequencing hold greater promise as third-generation sequencing technologies [41, 45, 46]. The generation of millions of tags at low cost makes these technologies the system of choice for gene expression analysis. A reduction in the cost of sequencing services, which is expected to happen in the near future, will have a major positive impact not only on gene expression studies but also on molecular breeding in agri-hort crops and tree species.

12.3 Metabolomics

Metabolomics is considered the ultimate level of postgenomic analysis as it can reveal changes in metabolite fluxes that are controlled by only minor changes within gene expression measured using transcriptomics and/or by analyzing the proteome that elucidates posttranslational control over enzyme activity [47, 48]. Metabolome refers to a set of metabolites that are formed within a biological system and their types and levels can be regarded as the ultimate response of biological systems to genetic or environmental changes [47]. Central to metabolomics is a range of metabolite fingerprinting and profiling technologies and extraction methods, which profile an entire extract without bias; the richest metabolite profiles will most easily be obtained by employing a range of extraction methods and analytical instruments due to the fact that none is without bias toward certain groups of compounds. Hence, metabolomics is the study of final downstream product of a genomic response as the total quantitative collection of small molecular weight compounds (metabolites) present in a cell/tissue type to whole organism [49]. Metabolomics as of now is considered a

technically demanding interdisciplinary research field that requires expertise in the fields of biology, analytical chemistry, organic chemistry, chemometrics, and informatics sciences. Metabolomic analysis consists of three distinct experimental parts: (a) preparation of the sample, (b) acquisition of data using analytical chemical methods, and (c) data mining using appropriate chemometric methods [47]. Essentially, all these steps are strongly interrelated and interdependent.

Two main metabolite profiling strategies are (i) mass spectrometry (MS) and (ii) nuclear magnetic resonance (NMR). The gas chromatography–mass spectrometry (GC-MS), gas chromatography–time-of-flight mass spectrometry (GC-TOF-MS), and liquid chromatography–mass spectrometry (LC-MS) are extensively used MS-based techniques in metabolite analyses. The GC-MS technology enables the identification and quantification of over a few hundred primary metabolites within a single extract [47, 50]. The GC-TOF-MS offers fast scan times, resulting in an improved peak deconvolution and higher sample throughput. On the other hand, LC-MS measures a far broader range of metabolites including primary and secondary metabolites [51]. In addition to this, capillary electrophoresis–mass spectrometry (CE-MS) and fourier-transformation cyclotron resonance–mass spectrometry (FT-ICR-MS) are also used. CE-MS is considered a highly sensitive methodology to detect low-abundance metabolites in plant samples [47]. The FT-ICR-MS relies solely on very high-resolution mass analysis, which potentially enables the measurement of the empirical formula for thousands of metabolites, although it is somewhat limited by the lack of chromatographic separation. NMR approaches rely on the detection of magnetic nuclei of atoms after application of a constant magnetic field for metabolite profiling [52]. NMR can provide subcellular information and it is easier to derive atomic information for flux modeling from NMR than from MS-based approaches [47]. In plant systems, metabolomics approach has already been used to study metabolomic changes during a variety of stresses, for example, temperature [53], water and salinity [54], sulfur [55], phosphorus [56], and oxidative [57] and heavy metals [58]. These tools have recently been turned to evaluation of the natural variance apparent in metabolite composition. Metabolomics approaches have great value in both phenotyping and diagnostic analyses in plants that might eventually enable metabolomics-assisted breeding in crop plants [59].

12.4 Proteomics

Proteomics is the qualitative and quantitative comparison of proteomes under different conditions to further unravel biological processes. The term proteome refers to all proteins expressed by a genome in the targeted tissues at a defined time point. It encompasses a broad range of tools and techniques in determining the identity and quantity of the expressed proteins in cells/tissues, their 3D structure, and other interacting partners that help to disclose gene function. Changes that occur at the protein level can be traced to genetic sequences, thus forming a unique cross reference to the complex biological phenomenon being investigated. This involves

separation, identification, determination of function, and its interactions with other proteins and biological molecules [60]. Protein profiling techniques allow a rapid comparison of complex samples and direct investigation of tissue specimens. In addition, proteomics has been complemented by the analysis of posttranslational modifications and techniques for the quantitative comparison of different proteomes [60, 61].

Techniques such as matrix-assisted laser desorption/ionization (MALDI) has been employed for rapid determination of proteins in particular mixtures besides electrospray ionization (ESI). Most proteins function in collaboration with other proteins, and one goal of proteomics is to identify which proteins interact. This is especially useful in determining potential partners in cell signaling cascades [60]. There has been extensive research over the last few years to study the technical aspects of proteomics in plants [62] and studies have been conducted in *Arabidopsis*, rice [63]), maize [64], barley [65], and chickpea [66]. Proteomics not only enables the study of protein–protein interaction but also helps in identification of multisubunit complexes [67]. Furthermore, proteomics can act as a powerful approach to organize and identify the proteome through development of 2DE gel protein reference maps of subproteomes in different plant species.

12.5 Interactomics

The complexity of an organism or even a complex trait of an organism cannot be completely explained by mere total number of genes alone. The organism would utilize combinatorial complexity to manifest required form of growth and/or resources in time and space [5, 68]. Therefore, to elucidate the complete functioning of an organism, one not only need to learn the biochemical function(s) of every protein and every domain but also need to discover all protein–protein interactions: developing an interactome is a prerequisite to understand the complex web of interactions that link biological molecules in a cell [3, 4, 69]. The network of all interactions is called the interactome that thus aims to compare such networks of interactions. The interactome maps are important in defining gene function and understanding the function of macromolecular complexes. Various experimental approaches for creating large-scale protein–protein interaction maps in plants have been reviewed by Morsy *et al.* [70].

The study of the interactome requires collection of a large amount of data from a single organism under a small number of perturbations. The two-hybrid screening (Y2H), tandem affinity purification, X-ray tomography, and optical fluorescence microscopy are generally employed for this purpose [71]. High-throughput versions of some of these methods have already been developed, although there is room for further improvement. It is envisaged to combine microarrays and mass spectrometry to enhance throughput of the technique. Tandem affinity purification (TAP) is a technique for studying protein–protein interactions. It involves creating a fusion protein with a designed piece, the TAP tag, on the end. The protein of interest with the

TAP tag first binds to beads coated with IgG, the TAP tag is then broken apart by an enzyme, and finally a different part of the TAP tag binds reversibly to beads of a different type [72]. After the protein of interest has been washed through two affinity columns, it can be examined for binding partners. Interactomic approaches are increasingly becoming relevant to gain a comprehensive understanding of both basic and applied aspects of complex plant–environment interactions [35, 73].

12.6 Genomics (or High-Throughput Genotyping) and Phenomics

The omic strategies, as mentioned above, can highlight candidate genes, metabolites, proteins, and so on that are responsible for a particular phenotype [74, 75]. Importantly, this information can be used in applied breeding programs through molecular marker technologies [8, 76]. Although many kinds of marker technologies have been developed, at present, simple sequence repeats (SSRs) or microsatellite, diversity array technologies (DArT), and SNPs are the marker systems of choice [37, 77–80]. Among these systems, SNP and DArT marker systems can be used for very high-throughput genotyping.

Several high-throughput SNP genotyping platforms are available with varying levels of suitability in practical use. Two platforms, namely, Illumina's GoldenGate assay and whole-genome genotyping Infinium assay, hold practical significance. In the former platform, the genomic DNA is activated using paramagnetic particles and PCR-amplified using three oligos and a universal PCR primer pair for each SNP. Two of the oligos used are allele-specific oligos that, upon ligation to the target allele, extends and ligates to the third locus-specific oligo (LSO) that contains SNP-specific tag and sequence complementary to the universal primer. The universal primer carries allele-specific fluorescent label and contains an address sequence that helps in binding the amplified product to the beads of fiber optic array. Genotyping is done in multiple of 96. GoldenGate assays have been developed for several crop species such as barley [81], wheat [82], maize [83], and common bean [84]. SNP genotyping based on GoldenGate assay has been very successful in constructing a genetic map and trait mapping [81, 84]. Crop-specific efforts are at different levels of success in developing first-generation GoldenGate assays for SNP genotyping worldwide. It is expected that in the next couple of years, the SNP GoldenGate assays would be available and large-scale use of SNPs will become integral to genetics and breeding efforts in most of the crop plants [9, 43].

The whole-genome profiling Infinium assay is done through comparative genomic hybridization. The change in the allele composition is measured through varying signal intensities. This assay includes whole-genome amplification to increase the amount of DNA followed by fragmentation and capturing onto bead array through SNP-specific primer. The primer anneals adjacent to a SNP and extension takes place that involves incorporation of hapten-labeled nucleotide corresponding to the SNP allele. Incorporated hapten-labeled nucleotides are detected by adding fluorescent-labeled antibodies during various steps to amplify the signal. Development of Infinium assays is in progress for some crop species such as soybean, maize, and so on.

The advent of a number of omics technologies and especially high-throughput genotyping has now made phenotyping the priority in crop research. Phenotypes that can be studied across species are more attractive, particularly given the rapid development in transgenic modeling. With advances in high-throughput genotyping technologies, the rate-limiting step of large-scale genetic investigations has been the accurate high-throughput phenotyping in a large number of samples. Phenomics is an emerging transdiscipline dedicated to the systematic study of phenotypes on a genome-wide scale. It is the systematic measurement and analysis of qualitative and quantitative traits, including clinical, biochemical, and imaging methods, for the refinement and characterization of a phenotype. Phenomics require deep phenotyping, the collection of a wide breadth of phenotypes with fine resolution, and phenomic analysis composed of constructing heat maps, cluster analysis, text mining, and pathway analysis (Figure 12.1). Many technologies have been developed to help explain the phenotypic consequences of genetic and/or environmental modifications in areas such as functional genomics, pharmaceutical research, and metabolic engineering [85–87]. The advances in metabolomics

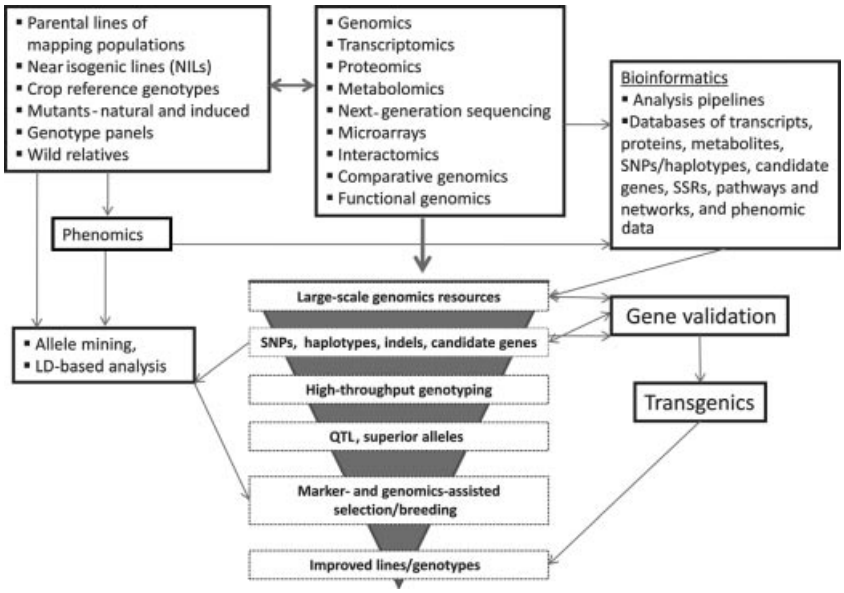

Figure 12.1 An overview of omics technologies and their applications in crop research and breeding. The omics platforms have potential to generate large-scale genomic resources, protein, and metabolite profiles from specific tissues that on their own and in combination with parallel but converging platforms can aid in pinning down the superior alleles, QTL, and candidate genes, besides contributing towards a deeper understanding of complex traits for crop improvement. Recent advances in these platform technologies and the bioinformatic pipelines have led to an accelerated development of robust and high-throughput marker systems. These advancements have ushered in new opportunities and strategies in crop research and breeding of even orphan crops.

and proteomics have a direct bearing on the large-scale phenotyping with a greater sensitivity in a high-throughput manner.

12.7 Integrated Omics Technology Approach

Availability of a number of omics technologies coupled with the vast quantity of genotyping data and volumes of precise phenotyping data opens an opportunity for displaying these techniques in an integrated approach in crop research and breeding [8, 10, 37, 42, 88]. This approach is expected to aid in gaining a better understanding of complex traits and environmental interactions [6]. This would be true both at cellular and whole-plant/crop level. Single-cell analysis was once considered beyond the capacity of omics technologies, but the recent examples of single-cell genomics, transcriptomics, proteomics, and metabolomics indicate an accelerated change largely owing to the rapidly emerging technologies that range from micro/nanofluidics to microfabricated interfaces for mass spectrometry to second- and third-generation automated, high-precision DNA sequencers. Such integration will enable the identification of genes and gene products, and can elucidate the functional relationships between genotype and observed phenotype, thereby permitting a system-wide analysis from genome to phenome, enabling accurate trait mapping, introgression of superior alleles, and in some cases the cloning of major QTL [88] for hitherto considered complex characters such as abiotic and biotic stress tolerance (Figure 12.1; see also Refs [89, 90]). Results of such an integration of omics technologies in model systems and selected crops is highly encouraging. Within next half a decade or so, omics technologies should be available for crop research and breeding in most of the crop plants.

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References

1 Holmes, C., McDonald, F., Jones, M. *et al.* (2010) *OMICS.*, **14**, 327–332.
2 North, H., Baud, S., Debeaujon, I. *et al.* (2010) *Plant J.*, **61**, 971–981.
3 Hecker, M., Lambeck, S., Toepfer, S. *et al.* (2009) *Biosystems.*, **96**, 86–103.
4 Long, T.A., Brady, S.M., and Benfey, P.N. (2008) *Annu. Rev. Cell Dev. Biol.*, **24**, 81–103.
5 Sauer, U., Heinemann, M., and Zamboni, N. (2007) *Science*, **316**, 550–551.
6 Bohnert, H.J., Gong, Q., Li, P. *et al.* (2006) *Curr. Opin. Plant Biol.*, **9**, 180–188.

7 Abuqamar, S., Luo, H., Laluk, K. *et al.* (2009) *Plant J.*, **58**, 347–360.
8 Edwards, D. and Batley, J. (2010) *Plant Biotechnol. J.*, **8**, 2–9.
9 Varshney, R.K. and Dubey, A. (2009) *J. Plant Biochem. Biotechnol.*, **18**, 127–138.
10 Yano, M. and Tuberosa, R. (2009) *Curr. Opin. Plant Biol.*, **12**, 103–106.
11 Costa, V., Gallo, M.A., Letizia, F. *et al.* (2010) *PPAR Res.* DOI: 10.1155/2010/409168.
12 Harbers, M. and Carninci, P. (2005) *Nat. Methods.*, **2**, 495–502.
13 Mardis, E.R. (2008) *Trends Genet.*, **24**, 133–141.
14 Czechowski, T., Bari, R.P., Stitt, M. *et al.* (2004) *Plant J.*, **38**, 366–379.
15 Schena, M., Shalon, D., Davis, R.W. *et al.* (1995) *Science*, **270**, 467–470.
16 Sreenivasulu, N., Kishor, P.B.K., Varshney, R.K. *et al.* (2002) *Curr. Sci.*, **83**, 965–973.
17 Sreenivasulu, N., Sunkar, R., Wobus, U. *et al.* (2010) *Methods Mol. Biol.*, **639**, 71–93.
18 Gupta, P.K., Roy, J.K., and Prasad, M. (1999) *Curr. Sci.*, **77**, 875–884.
19 Buchanan, C.D., Lim, S., Salzman, R.A. *et al.* (2005) *Plant Mol. Biol.*, **58**, 699–720.
20 Oztur, Z.N., Talame, V., Deyholos, M. *et al.* (2002) *Plant Mol. Biol.*, **48**, 551–573.
21 Rabbani, M.A., Maruyama, K., Abe, H. *et al.* (2003) *Plant Physiol.*, **133**, 1755–1767.
22 Rensink, W.A., Iobst, S., Hart, A. *et al.* (2005) *Funct. Integr. Genomics.*, **5**, 201–207.
23 Seki, M., Carninci, P., Nishiyama, Y. *et al.* (1998) *Plant J.*, **15**, 707–720.
24 Seki, M., Narusaka, M., Ishida, J. *et al.* (2002) *Plant J.*, **31**, 279–292.
25 Seki, M., Narusaka, M., Abe, H. *et al.* (2001) *Plant Cell.*, **13**, 61–72.
26 Legay, S., Lamoureux, D., Hausman, J.F. *et al.* (2009) *Plant Cell Rep.*, **28**, 1799–1816.
27 Flavell, A.J., Bolshakov, V.N., Booth, A. *et al.* (2003) *Nucleic Acids Res.*, **31**, e115.
28 Hou, P., Ji, M., Li, S. *et al.* (2004) *Clin. Chem.*, **50**, 1955–1957.
29 Conesa, A., Forment, J., Gadea, J. *et al.* (2007) Microarray technology in agricultural research, in *Microarray Technology Through Application*, Taylor & Francis, Abingdon, pp. 173–209.
30 Adams, M.D., Kelley, J.M., Gocayne, J.D. *et al.* (1991) *Science*, **252**, 1651–1656.
31 Gowda, M., Jantasuriyarat, C., Dean, R.A. *et al.* (2004) *Plant Physiol.*, **134**, 890–897.
32 Velculescu, V.E., Zhang, L., Vogelstein, B. *et al.* (1995) *Science*, **270**, 484–487.
33 Brenner, S., Johnson, M., Bridgham, J. *et al.* (2000) *Nat. Biotechnol.*, **18**, 630–634.
34 Meyers, B.C., Vu, T.H., Tej, S.S. *et al.* (2004) *Nat. Biotechnol.*, **22**, 1006–1011.
35 Torto, T.A., Li, S., Styer, A. *et al.* (2003) *Genome Res.*, **13**, 1675–1685.
36 Sun, M., Zhou, G., Lee, S., Chen, J., Shi, R.Z., and Wang, S.M. (2004) *BMC Genomics*, **5**, 1.
37 Joosen, R.V., Ligterink, W., Hilhorst, H.W. *et al.* (2009) *Curr. Genomics*, **10**, 540–549.
38 Gowda, M., Venu, R.C., Raghupathy, M.B. *et al.* (2006) *BMC Genomics*, **7**, 310.
39 Gowda, M., Li, H., and Wang, G.L. (2007) *Nat. Protoc.*, **2**, 1622–1632.
40 Porreca, G.J., Zhang, K., Li, J.B. *et al.* (2007) *Nat. Methods*, **4**, 931–936.
41 Gupta, P.K. (2008) *Trends Niotechnol.*, **26**, 1135.
42 Gupta, P.K., Rustgi, S., and Mir, R.R. (2008) *Heredity*, **101**, 5–18.
43 Varshney, R.K., Nayak, S.N., May, G.D. *et al.* (2009) *Trends Biotechnol.*, **27**, 522–530.
44 Hudson, M. (2008) *Mol. Ecol. Resour.*, **8**, 3–17.
45 McCarthy, A. (2010) *Chem. Biol.*, **17**, 675–676.
46 Schadt, E.E., Turner, S., and Kasarskis, A. (2010) *Hum. Mol. Genet.*, **19** (R2), R227–R240.
47 Keurentjes, J.J. (2009) *Curr. Opin. Plant Biol.*, **12**, 223–230.
48 Saito, N., Robert, M., Kitamura, S. *et al.* (2006) *J. Proteome Res.*, **5**, 1979–1987.
49 Kim, H.K. and Verpoorte, R. (2010) *Phytochem. Anal.*, **21**, 4–13.
50 Roessner, U., Luedemann, A., Brust, D. *et al.* (2001) *Plant Cell.*, **13**, 11–29.
51 Tohge, T., Nishiyama, Y., Hirai, M.Y. *et al.* (2005) *Plant J.*, **42**, 218–235.
52 Fernie, A.R., Trethewey, R.N., Krotzky, A.J. *et al.* (2004) *Nat. Rev. Mol. Cell Biol.*, **5**, 763–769.
53 Kaplan, F., Kopka, J., Sung, D.Y. *et al.* (2007) *Plant J.*, **50**, 967–981.
54 Cramer, G.R., Ergul, A., Grimplet, J. *et al.* (2007) *Funct. Integr. Genomics*, **7**, 111–134.
55 Nikiforova, V.J., Daub, C.O., Hesse, H. *et al.* (2005) *J. Exp. Bot.*, **56**, 1887–1896.

56 Hernandez, G., Ramirez, M., Valdes-Lopez, O. *et al.* (2007) *Plant Physiol.*, **144**, 752–767.

57 Baxter, C.J., Redestig, H., Schauer, N. *et al.* (2007) *Plant Physiol.*, **143**, 312–325.

58 Le Lay, P., Isaure, M.P., Sarry, J.E. *et al.* (2006) *Biochimie*, **88**, 1533–1547.

59 Fernie, A.R. and Schauer, N. (2009) *Trends Genet.*, **25**, 39–48.

60 Schulze, W.X. and Usadel, B. (2010) *Annu. Rev. Plant Biol.*, **61**, 491–516.

61 Hirsch, J., Hansen, K.C., Burlingame, A.L. *et al.* (2004) *Am. J. Physiol. Lung Cell Mol. Physiol.*, **287**, L1–L23.

62 van Wijk, K.J. (2001) *Plant Physiol.*, **126**, 501–508.

63 Tsugita, A., Kamo, M., Kawakami, T. *et al.* (1996) *Electrophoresis.*, **17**, 855–865.

64 Chang, W.W., Huang, L., Shen, M. *et al.* (2000) *Plant Physiol.*, **122**, 295–318.

65 Witzel, K., Weidner, A., Surabhi, G.K. *et al.* (2010) *Plant Cell Environ.*, **33**, 211–222.

66 Pandey, A., Choudhary, M.K., Bhushan, D. *et al.* (2006) *J. Proteome Res.*, **5**, 3301–3311.

67 Washburn, M.P., Wolters, D., and Yates, J.R., 3rd (2001) *Nat. Biotechnol.*, **19**, 242–247.

68 Cesareni, G., Ceol, A., Gavrila, C. *et al.* (2005) *FEBS Lett.*, **579**, 1828–1833.

69 D'Alessandro, A., Zolla, L., and Scaloni, A. (2011) *Mol. Biosyst.*, **7** (3), 889–898.

70 Morsy, M., Gouthu, S., Orchard, S. *et al.* (2008) *Trends Plant Sci.*, **13**, 183–191.

71 Chamousset, D., Mamane, S., Boisvert, F.M. *et al.* (2010) *Proteomics.*, **10**, 3045–3050.

72 Xu, X., Song, Y., Li, Y. *et al.* (2010) *Protein Expr. Purif.*, **72**, 149–156.

73 Gonzalez-Fernandez, R., Prats, E., and Jorrin-Novo, J.V. (2010) *J. Biomed. Biotechnol.*, 932527.

74 Shulaev, V., Cortes, D., Miller, G. *et al.* (2008) *Physiol. Plant.*, **132**, 199–208.

75 Tuberosa, R., Gill, B.S., and Quarrie, S.A. (2002) *Plant Mol. Biol.*, **48**, 445–449.

76 Varshney, R.K. and Tuberosa, R. (2007) Genomics-Assisted Crop Improvement: An Overview in *Genomics Applications in Crops*, vol. **2**, Springer, Dordrecht, pp. 1–12.

77 Comadran, J., Thomas, W.T., van Eeuwijk, F.A. *et al.* (2009) *Theor. Appl. Genet.*, **119**, 175–187.

78 Grewal, T.S., Rossnagel, B.G., Pozniak, C.J. *et al.* (2008) *Theor. Appl. Genet.*, **116**, 529–539.

79 Jimenez-Gomez, J.M. and Maloof, J.N. (2009) *BMC Plant Biol.*, **9**, 85.

80 Wenzl, P., Suchankova, P., Carling, J. *et al.* (2010) *Theor. Appl. Genet.*, **121**, 465–474.

81 Rostoks, N., Mudie, S., Cardle, L. *et al.* (2005) *Mol. Genet. Genomics*, **274**, 515–527.

82 Akhunov, E., Nicolet, C., and Dvorak, J. (2009) *Theor. Appl. Genet.*, **119**, 507–517.

83 Barbazuk, W.B., Emrich, S.J., Chen, H.D. *et al.* (2007) *Plant J.*, **51**, 910–918.

84 Hyten, D.L., Song, Q., Fickus, E.W. *et al.* (2010) *BMC Genomics.*, **11**, 475.

85 Bilder, R.M., Sabb, F.W., Cannon, T.D. *et al.* (2009) *Neuroscience.*, **164**, 30–42.

86 Ihlow, A., Schweizer, P., and Seiffert, U. (2008) *BMC Plant Biol.*, **8**, 6.

87 Jewett, M.C., Hofmann, G., and Nielsen, J. (2006) *Curr. Opin. Biotechnol.*, **17**, 191–197.

88 Salvi, S. and Tuberosa, R. (2007) Cloning QTLs in Plants, in *Genomics-Assisted Crop Improvement: Genomics Approaches and Platforms*, vol **1**, Springer, Dordrecht, pp. 207–226.

89 Collins, N.C., Tardieu, F., and Tuberosa, R. (2008) *Plant Physiol.*, **147**, 469–486.

90 Xue, S., Li, G., Jia, H. *et al.* (2010) *Theor. Appl. Genet.*, **121**, 147–156.

13
The Use of "Omics" Approaches in *Arabidopsis* for the Improvement of Abiotic Stress Tolerance

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The recent progress in "omics" approaches, which allow the identification and quantification of hundreds to thousands of biological molecules in a single sample, has allowed their wide application in the plant sciences. Their use has permitted the detailed characterization of the developmental and environmental factors that control plant responses to abiotic stress and provided a basis upon which to base approaches to improve plant stress tolerance. This chapter reviews relevant aspects of the "omics" technologies themselves and highlights the main considerations in getting from a plant sample to analyzed data. As the majority of fundamental plant research uses *Arabidopsis* as a model plant, we restrict this chapter to review examples of how the application of "omics" approaches has allowed the characterization of abiotic stress responses and the use of these data to support early discovery research to engineer plants with improved stress tolerance.

13.1
Introduction

Over the past decade, there has been an unprecedented progress in technologies to allow a detailed molecular characterization of organisms. These so-called "omics" approaches, including, to name but a few, transcriptomics, proteomics, and metabolomics, have been applied on a broad scale to numerous aspects of plant biology and have fueled the development of plant systems biology. These technologies allow identification or quantification of hundreds to thousands of molecules in a single biological sample. While previous approaches studied single or a few molecules, analysis on a much more detailed level has now become possible. The biological questions (or samples) to which these have been applied are familiar from the fields of developmental biology and environmental responses, and have increasingly incorporated genetic, temporal, and spatial resolutions.

Plants must survive and reproduce in the environment in which they grow, yet these environments are changeable and thus may often become suboptimal. These so-called abiotic stress conditions, such as drought or temperature extremes,

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substantially affect plant growth and development and lead to major crop yield losses. Therefore, there is a considerable interest in improving fundamental knowledge of plant–environment interactions and leveraging of this knowledge to generate crops with improved yield under abiotic stress conditions in the field. As with most basic plant science, *Arabidopsis* is the model plant of choice for most laboratories and additionally can represent an intermediate testing phase in the process to improve abiotic stress tolerance in crop species.

The combination of the technological advances in "omics" approaches and the considerable interest in plant abiotic stress tolerance means there is an increasing body of knowledge that is of potential direct or indirect relevance to efforts to improve plant abiotic stress tolerance. While other chapters consider crop species directly, we will restrict ourselves to review the relevant progress and knowledge in *Arabidopsis*. We will thus briefly review relevant aspects of available "omics" technologies and the resulting data analysis before describing the application of these technologies in fundamental *Arabidopsis* abiotic stress research and finally discussing how this knowledge can be used in applied early discovery research to improve plant tolerance to abiotic stress.

13.2 "Omics" Approaches

13.2.1 Genomics

Genomics should be regarded as the enabler and namesake of those technologies that (rightly or wrongly) today have "omics" as a suffix and that will be discussed in this chapter. In plant science, the completion of the *Arabidopsis* genome sequence [1] allowed the emergence of the era of "postgenomics" whereby theoretically the entire catalog of transcripts, proteins, and (indirectly via encoded enzymes) metabolites could be discovered. It was upon this base that subsequent technological developments in "omics" approaches became possible. Furthermore, "omics" approaches are often described as the analysis of subsequent layers of information with respect to the fields of genomics, transcriptomics, proteomics, and metabolomics, as they become closer to the observed whole-plant phenotype (also called "phenomics"). Although it is these "postgenomic" technologies that are predominantly being referred to as "omics" approaches, genomics is worth highlighting here due to its integral place in the overall field of plant systems biology and the fact that with the advent of new-generation sequencing tools its importance is set to increase further. The increase in sequencing capabilities is well known and described [2], and for the purpose of this chapter it is sufficient to say that the sequencing of DNA (or RNA (transcriptomics) or modified DNA (epigenomics)) is becoming much faster and much cheaper than ever before. This makes the sequencing of genomes (species, accessions, cultivars, mutants, etc.), previously taking years of effort for a worldwide consortium, possible in days for a single

researcher to accomplish. In this context, approaches that will likely have a particular impact in the field of plant abiotic stress research will include the mapping of mutations [3], comparative genomics of *Arabidopsis* accessions, stress-tolerant relatives, and diverse plant species.

13.2.2 Transcriptomics

Of all "omics" approaches, transcriptomics has been by far the most widely applied. This was predominantly because of the availability of microarray technology that was best able to leverage the completed genome sequence, although high-throughput quantitative PCR (QPCR) and increasingly direct sequencing are also important. Microarrays are essentially glass slides that have thousands of DNA spots attached to them allowing the detection of the corresponding transcripts within a given sample. As knowledge on all known or predicted transcripts became available, they were able to be rapidly included in in-house or commercial microarrays making the "genome-wide" quantification of transcripts routine. Historically, the most popular platform has been the Affymetrix ATH1 array, for which there are now data from more than 10 000 biological samples in the public domain [4], while for other platforms, for example, the commercial Agilent and the academic CATMA array [5], there are thousands more deposited in public databases. Microarrays are still in common use, aided by the development of more sophisticated and comprehensive microarrays such as the AGRONOMICS1 Tiling array [6], but their dominance is already being challenged by sequencing-based approaches.

It should be noted that microarrays, common to any sequence hybridization-based technology, have technical limitations relating to sensitivity and specificity. It is therefore possible to detect only "known" transcripts (i.e., those sequences included in the array), which are within the dynamic (quantifiable) range of the microarray, and it may not be possible to distinguish closely related transcripts. QPCR is more sensitive and was the method of choice for the validation of microarray results; however, due to technical robustness of commercial microarrays, it is now viewed to be better to have biological validation of results (i.e., independent experiments) rather than technical repetitions (i.e., duplicate arrays) or validation (via QPCR, Northern, etc.) on the same RNA samples. Combined with automation, QPCR has also emerged as a useful platform to quantify focused subsets of genes, for example, transcription factors [7]. Other technical platforms have recently emerged that offer similar sensitivity to QPCR, notably the nanostring technology [8], although these have not yet been adopted on a broad scale. All of these technologies have in common the need for *a priori* knowledge on transcript identity. As sequencing costs have come down, it is therefore becoming increasingly attractive to directly sequence transcripts in a given biological sample. The most notable benefits are that knowledge on transcript identity is not required and the discrimination of closely related transcripts or splice variants can be improved. It should nonetheless be noted that without a

genome sequence the downstream data analysis may be prohibitively difficult or even not possible in the case of some "tag"-based methods (i.e., cutting a specific sequence) that require sequence information. Furthermore, the discussion on sequence bias is still relevant as because most sequencing technologies utilize an amplification step (i.e., requiring sequence hybridization) they will have sequence bias in transcript detection, although it is hoped that direct sequencing [9] may in the future overcome this. In summary, transcriptomics has become a routine part of plant molecular biology through the application of microarrays and QPCR, and the advent of new-generation sequencing-based methods means that there will likely be further interesting developments in the near future.

13.2.3 Proteomics

Proteomics not only refers to the measurement of cellular protein but may also encompass the study of modifications (notwithstanding, other terms may be used, for example, phosphoproteomics). Protein analysis is more challenging than for transcripts due to the more diverse structural properties of proteins (e.g., membrane bound, chemically modified, or resistant to trypsin digestion) in comparison to mRNA. Methodologies generally use some form of chromatographic separation, followed by protein identification by on- or offline mass spectrometry (MS), usually of peptides derived from proteolytic digestion (reviewed by Wienkoop *et al.* [10]). The measured peptides are mapped to the corresponding proteins using sequence information, and there are different approaches that can be used for the (semi)quantification of the corresponding proteins. In general, the peptides that map to a specific gene can be counted and the total count can indicate the abundance of the corresponding protein. Alternatively, and more quantitatively, differential labeling of the proteins contained in different biological samples may be used. This can be utilized at the level of the biological samples before harvest by supplying isotopically labeled metabolites during their growth [11], but the need to fully label samples should be considered as this can present limitations or difficulties in experimental design and plant growth conditions. For example, most labs favor the use of liquid culture as few have the specialized equipment necessary to control the CO_2 atmosphere, or the use of young seedlings may be compromised by the presence of unlabeled isotopes in seed reserves. It is therefore unsurprising that methods have been developed to allow differential labeling of samples postharvest using isotope-coded covalent tags (e.g., iTRAQ). In either approach, samples can be combined and analyzed in parallel and the differential mass of the resulting peptides can be determined via MS and used to derive accurate ratios of protein abundance in the samples. Despite such methodological and technological advances, proteomics remains challenging and time consuming meaning that it is the "omics" approach that is least frequently applied. However, despite this the importance of studying changes at the protein level is becoming increasingly recognized and no doubt its use will become more widespread.

13.2.4 Metabolomics

Metabolite profiling was adopted early with respect to the plant sciences and there are many excellent reviews [12, 13]. Generally, metabolomics refers to the simultaneous quantification of multiple metabolic compounds (e.g., primary and secondary metabolites, plant hormones, etc.) in a given biological sample. Owing to the diverse chemical nature of the substances and the greater dynamic range of their abundance, it is more technically difficult to quantify them (at a global level) than transcripts or proteins. In common to proteins, approaches usually couple chromatography-based separation followed by MS. The most widely used is gas chromatography MS (GC-MS) that routinely allows the detection of a few hundred metabolites, mostly from primary metabolism. Liquid chromatography has been mostly applied to secondary metabolites or to specific classes of metabolites, such as amino acids. Recently, advances in technology based on Fourier transform MS (FT-MS), which allow very high mass accuracy (i.e., sufficient to determine the atoms comprising a molecule), have enabled broader profiling of several metabolite classes [14]. In addition to improvements in MS technologies, the chromatography has also improved with the availability of nano-HPLC and UPLC allowing shorter run times and better resolution. Similar to proteomics, differential isotopic labeling can be used to quantify ratios of metabolites between samples, although it is more common to use chromatographic peak area or height to determine abundance [13]. Metabolic profiling is affected by "ion suppression," whereby the presence of one molecule interferes with the detection/quantification of another, which increases in relevance when little or no chromatographic separation is used. An elegant methodology to overcome this has been the use of an istopically labeled full metabolome as a "standard" that is included in each sample and against which accurate ratios can be derived to compare biological samples [14]. Taken together, metabolic profiling technologies are today a powerful approach able, in combination, to measure several hundred metabolites in a biological sample of interest; however, limitations in sample quantity, time, and resources mean that often only a subset of these possible metabolites is measured.

13.2.5 Other "Omics" Approaches

The general term "-ome" and the derived "omics" can seemingly be suffixed to any and all biological entities; however, for the purpose of this chapter, we will limit the scope to those more broadly adopted or studied by the community. There are several "omics" approaches that are based on adapted forms of those technologies already described for genomics or transcriptomics, that is, they measure modified DNA or noncoding RNAs/DNA/RNA associated with a certain protein. There are several relevant "omics"-type approaches in the field of epigenetics, which is important in the context of abiotic stress [15] and relates to the direct (e.g., methylation) or indirect (e.g., histone acetylation) modifications of DNA and the small RNA molecules that influence them. Several methodologies have been applied to study small RNAs,

including microRNAs, where their abundance can be measured in approaches analogous to those described for transcriptomics [16]. Second, genomic DNA can be treated with bisulfite or digested with methylation-sensitive restriction enzymes and combined with microarrays or sequencing to characterize DNA methylation (methylome) [16]. The next group of approaches uses chromatin immunoprecipitation (ChIP), which via the precipitation of specific proteins (or modified proteins) can be used to isolate DNA or RNA molecules that are associated with them [17]. This can be done using antibodies specific to modified histones to further characterize the "epigenome," or can be applied either to transcription factors (or any other nucleic acid binding protein), where the DNA binding sites can be identified [18], or to a specific ribosome (possibly expressed in a specific tissue) to identify transcripts being translated (i.e., "translatome") [19]. The "translatome" can also be identified by using density-based separation of polysomes and then characterizing the bound mRNA. Similarly, sequencing or microarrays can be directly applied to other RNA species, not just mRNA transcripts to detect their changes in response to stress. Other possibilities include the inhibition of transcription or translation combined with "omics" approaches in order to follow transcript turnover times or to see if a specific change occurs directly or rather indirectly via the synthesis of an intermediate protein [20].

If instead proteins are isolated rather than nucleic acid, then obviously proteomic approaches can be used to identify interacting proteins. This has been successfully applied on a large scale to obtain comprehensive interaction networks. Other methods have also been adapted to obtain protein–protein interaction data sets such as large-scale yeast two-hybrid screens or bimolecular florescence interaction that can yield large data sets. Collectively, such protein–protein data sets are increasingly referred to as the "interactome" [21].

Although the proliferation of "omics" has been most noticeable with respect to nucleic acids and proteins, there have been relevant developments in the study of small molecules. The most prominent of these has been the measurement of inorganic molecules that are important plant micro- or macronutrients, for which the term "ionomics" has been coined [22]. In summary, there are many "omics" approaches for the multilevel analysis of biological systems, which may be used either independently or in combination (i.e., integrated approaches) to investigate plant abiotic stress responses at the systems level.

13.3 Data Analysis

"Omics" approaches move beyond the study of single molecules as they generally quantify hundreds to thousands of molecules in parallel. However, as common to all biological experimentation, it is essential to note that their application and success are nonetheless based on good experimental design and appropriate data analysis. Many of these factors are well known and common to more classical experiments; however, there are also unique factors relating to the preprocessing and analysis of "omics" data that are unique and also worth highlighting in the following sections.

Top of the list for any biologist is good experimental design, for which randomization, blocking, and replication are essential considerations [23]. The variation in all factors beyond those being studied should be limited and controlled for at all stages of the experiment. Particularly, common sources of variation from environmental conditions such as temperature or light or temporal differences such as those across the day or following treatment should be closely monitored, minimized, and controlled for using appropriate experiment designs. It should be realized that variation in these factors can easily obscure the identification of the molecular basis of even seemingly drastic phenotypes or treatments. Furthermore, even if molecular changes are identified, their biological validity is ensured only by having independent biological repeats. Therefore, while pooling of plant material can help increase the number of sampled individuals, it is essential to have appropriate and sufficient biological replication from spatial or temporal repeats for statistical evaluation. The inclusion of "technical replicates" into classical statistical tests will always lead to overestimation of the significance of the observed changes.

The general approaches for primary data analysis will be covered in the following two sections either looking directly at molecular changes or considering "network analysis" based on correlation or association between molecules. The other two sections deal with the key steps of preprocessing (including normalization) of data prior to analysis and the visualization and analysis of the results from the primary analyses.

13.3.1 Data Preprocessing

Prior to analysis, all raw data from "omics" approaches require some form of preprocessing and/or normalization. The more intricate details of the diverse methods available for the various technologies are far beyond the scope of this chapter, but there are a few key considerations that are important to understand "omics" data and their limitations. First is the link between the data for a given sample and the biological molecules in that sample, that is, the "omics" data must be associated with the corresponding molecules. This is evidently relatively simple for transcriptomics but increases in complexity for proteomics and particularly metabolomics. However, even for transcriptomics, genome annotation can change over time and so the latest annotations should be sought and the potential ambiguities realized. The second key point is to ensure that different samples are comparable to each other – a process that additionally requires normalization. This is usually performed by using an algorithm that basically adjusts the "mean measurement signal" of all samples to the same value. This is very effective at removing technical variation that results from sample-to-sample variation in the labeling or detection of molecules. However, it is important to note that there can be actual biological variation in the total quantity of transcripts, proteins, or metabolites that will inevitably be removed by this process. This is best conceptualized by considering the effect on the total mRNA pool when a treatment causes a global inhibition of transcription [24], and while the absolute quantity of "all transcripts" will decline, a conventional microarray experiment assumes it does not. The only way to overcome

this is to include standards or "spiked" controls into the biological samples and to use these for normalization. Such approaches are not used in the vast majority of experiments and the possibility of such global changes are generally overlooked, although methods have been developed to control for this, particularly in transcriptomics [24, 25]. In general, the inclusion of appropriate external controls in "omics" experiments and the underlying assumption in data interpretation in their absence should be considered, particularly where a large proportion (e.g., >25%) of the measured molecules are described as changing.

13.3.2 Differential Abundance

The most common biological question addressed using "omics" approaches is to identify which molecules are changed in abundance in response to a given experimental factor; for example, which genes are induced by cold or drought? There is perhaps a surprising diversity of methods for how this question can be answered even for a single "omic" technology, let alone for diverse technologies. Although fold change and rank-based methods have and can be used, the most common and appropriate approaches are based on statistical evaluation of the comparisons of interest. This can be viewed similarly to the widely known *t*-test or ANOVA for comparison of two or more groups, respectively. However, mixed models have become the favored method for comparison due to their flexibility in evaluating experiments from the simplest design considering just a single factor (e.g., genotype A versus B) to complex designs incorporating several factors (e.g., genotype, + treatment across time) [23]. These models allow comparisons of biological interest to be easily made, such as the treatment or genotype effects, and allow a valid statistical comparison of the "interaction term" between two factors (rather than comparing lists from two tests). This area is most advanced for transcript analysis and there are many algorithms in the literature, the most favored of which "borrow" information across all molecules to increase statistical power over conventional statistics when only few replicates are used [26], an approach that has also been applied to proteomics data [27].

As generally "omics" approaches result in hundreds or thousands of tests being conducted simultaneously, the resulting *p*-values should be corrected for multiple testing. Methods that control the false discovery rate (fdr) are most popular as they allow biological conclusions to be drawn by accepting that a small (e.g., 0.01 or 0.05) proportion of the list of significant molecules will be wrong. Another often overlooked fact is that due to the low replication in many "omics" studies, the number of false negatives can be very high, which means that perhaps hundreds of molecules that are truly differentially abundant are not identified as significant.

13.3.3 Correlation and Network Analysis

Rather than comparing groups, it is also possible to use correlation-based (univariate or multivariate) approaches to identify transcripts, proteins, or metabolites of

biological interest. In general, this can be performed within a single "omics" data set, for example, looking at transcriptional correlation networks, or between multiple layers of information, such as between metabolites and transcripts or between "omics" data and an experimental (e.g., time) or physiological parameter of interest such as biomass or freezing tolerance [28]. Such approaches can be used to visualize molecules as "networks" or to group them into "modules" for further analysis. The use of correlation-based approaches, fueled by the large amount of data available, has been particularly evident for transcriptomics. Further developments have also incorporated other "omics" data such as interactome (protein–protein interactions) or transcription factor binding sites (*cis*-elements) in order to include other molecular relationships in such a "network." Additional information can also be utilized in various software algorithms to predict regulatory interactions (i.e., transcription factor – target gene relationships) to prioritize genes for functional studies [29, 30]. Similar correlation approaches have been used to associate metabolites and transcripts either to predict gene function [31, 32] or to reveal candidate metabolite signals [33]. The use of network approaches is an area of increasing interest and will no doubt lead to additional applications of relevance for the application of "omics" approaches.

13.3.4
Visualization and Comparative Analysis

The analysis of "omics" data usually results in the identification of a list of molecules of biological interest. For genes and proteins, it is useful to identify the associated molecular function or biological process, often referred to as gene ontology (GO) analysis or functional testing. In a more specific context, it is also useful to associate genes, proteins, and metabolites with the metabolic pathways in which they participate. There are many tools that are useful for GO analysis including BINGO [34] and MapMan [35] and PageMan [36]. MapMan and other tools such as KAPPA-view [37] and Aracyc [38] are able to visualize molecular changes displayed on biological pathways (or diagrams) of interest, which can be very useful for biological interpretation of complex sets of changes. Visualization of the regulation of specific genes is also aided by the development of comprehensive online tools such as the Botany Array Resource (BAR) with its popular Electronic Florescent Protein (eFP Browser) that allows tissue-, cell-, and treatment-specific gene expression changes to be visualized [39]. Furthermore, Genevestigator [40] allows a similar analysis where such differences can be visualized in graphs and heatmaps for desired genes of interest. Such tools are based on the growing abundance of public data sets, and it should be regarded that one of the main benefits of "omics" approaches has been the dissemination of thousands of data sets and the rise of computational biology. As mentioned in the preceding section, coexpression "network" analysis has been particularly successful and has benefited from data and numerous tools available online. The reader may refer to recent comprehensive reviews for both the applications (and limitations) of coexpression analysis [41] and the more general online data sources [42] on which they are mainly based. Finally, as online tools become increasingly comprehensive and integrate

multiple levels of "omics" data and allow the user to upload their own data and analyze them in the context of other data sets, for example, VirtualPlant [43], these tools will likely become more powerful for their biological application.

13.4 Characterization of Environmental Responses

Since plants are unable to escape a changing environment, plants reprogram their metabolism, growth, and development. Abiotic stress is defined as a nonliving environmental factor such as cold, drought, and heat of a potentially harmful nature. Such stressful conditions are rapidly perceived and trigger adaptation responses in plants to minimize the damage and eventually secure reproduction. In the literature, plant stress responses are often characterized using "avoidance–tolerance" models [44]. For example, osmotic stress that is common to freezing, salinity, or drought conditions will trigger osmolyte production helping plants to maintain low water potential and thus avoid dehydration while minimizing the effects of harmful reactive oxygen species (ROS) by producing protective proteins and antioxidants. These often vast physiological alterations are mediated by complex signaling pathways and are associated with changes on every "-ome" level (reviewed, for example, in Refs [45, 46]. During the last decade, functional genomics technologies have been used extensively to understand this complexity, and despite a significant progress it has become clear that stress responses are more intricate than initially expected. At first, many of the experiments focused on whole plants or mature organs and using rather severe or lethal stress conditions [47–50]. The obtained data provided a glimpse into stress expression profiles revealing hundreds of responsive genes, including key regulators, for example, class of DREB transcription factors and downstream genes such as those encoding (LEA) proteins, heat shock proteins, glutathione-*S*-transferases, and the biosynthetic enzymes of compatible solutes. The intricate hormonal regulation, including a central role for abscisic acid, became obvious. Metabolite profiling delineated a set of stress-responsive compounds, for example, galactinol and amino acid proline (reviewed by Seki *et al.* [51]), whereas proteomics studies revealed major changes in primary plant metabolism [52–55]. As expected, while some of the responses, for example, activation of ROS scavenging and repair mechanisms [48, 56], are shared between different stresses, others such as accumulation of heat stress proteins are condition specific [57]. It also became clear that stress severity, duration, time of the day, or tissue specificity can have a dramatic effect on molecular responses [19, 58–62], consistent with the fact that physiological outcome has to be precisely tailored to the surrounding environment. For example, severe drought stress is often accompanied by a collapse of photosynthesis and carbon starvation, which is in sharp contrast to moderate drought that, in fact, results in a favorable carbon balance, leading to very different molecular profiles [63]. In this chapter, we will focus on a number of recent -omics studies to demonstrate how the use of genetic variation, temporal and cellular resolution, and stress combination can help better understand plant stress responses.

13.4.1
The Use of Temporal Resolution

In a simplified view, stress imposition triggers a sequence of molecular events that will eventually translate into a new steady state and associated phenotype. Obviously, how this new steady state is reached is extremely interesting and can be addressed by introducing time component into study of interest. A comprehensive abiotic stress transcriptome data set obtained as part of the AtGeneExpress consortium will be used as an example [56]. Two weeks old plants grown in hydroponics were subjected to heat, cold, drought, salt, high osmolarity, UV-B light, and wounding. Roots and shoots of these plants were subsequently harvested at multiple time points (30 min, 1 h, 3 h, 6 h, 12 h, and 24) following stress imposition. Importantly, all treatments were done in parallel allowing direct comparison. Assisted by temporal resolution the authors could, for example, delineate an initial stress regulon that was common to all abiotic stimuli, whereas condition-specific responses developed only at the later time points. Transcripts that changed only transiently and those that stayed high or low across different time points could be identified. The obtained data were used by the authors and many other groups to generate hypotheses about causative relations between stress-responsive genes. For instance, the CERMT tool was developed taking into account the assumption that changes in transcription factor expression would precede those of downstream target genes [29]. Using simulated and real data, the authors could demonstrate that using multiple expression time series can provide reliable target gene lists for stress-responsive transcription factors. Time resolution was also successfully applied to dissect short- and long-term metabolic responses to salt stress using *Arabidopsis* cell cultures in combination with GC/MS and LC/MS technologies [64]. It is likely that predictions could be further improved by increasing the resolution of sampled time points, although this is at the moment hampered by the relatively high cost of profiling techniques.

13.4.2
The Use of Genetic Variation

Using genetic variation together with "omics" approaches is a powerful approach to dissect plant abiotic stress responses and several example cases will be given below. In the majority of cases, genetic variation is represented by loss- or gain-of-function mutants in genes associated with stress tolerance, which are then characterized alongside the corresponding wild-type plants under control and/or stress conditions. In this way, for instance, mutants in ABA synthesis and signaling assisted in discriminating between ABA-dependent and -independent transcripts and metabolites associated with drought stress [65]. Among other findings, the authors demonstrated that accumulation of proline, but not raffinose or galactinol, required functional ABA signaling. Omics approaches, particularly transcript profiling, have been also widely used to identify target genes of transcription factors central to stress responses such as CBF1–3, DREB2A, or ZAT12 [66–68]. In addition to mutants, another source of genetic variation in *Arabidopsis* is a natural variation encompassing

hundreds of different accessions that through evolution have adapted to specific, often very extreme environments. Thus, in contrast to mutant studies that are restricted to single or few genes, natural variation has the potential to provide information on how whole networks are adapted to particular stress stimuli. Significant variation in many phenotypic characteristics of *Arabidopsis* accessions including stress performance has been described in the literature and together with genetic information has provided the basis for the identification of genetic loci (QTL) responsible for the observed phenotypes (see Ref. [69] and references therein). Obviously, the underlying molecular basis of phenotypic variation can also be indirectly examined using molecular profiling. In an example study, natural variation in freezing tolerance among nine *Arabidopsis* accessions was correlated with metabolite and transcript changes to identify molecular changes that may be causally linked to differences in freezing tolerance [28]. Although only relatively few accession were used, the authors were able to delineate biological processes associated with freezing tolerance such as flavonoid biosynthesis, the CBF pathways, and downstream metabolites. A similar approach was used to identify candidate genes underlying physiological differences in drought adaptation between two extreme accessions Tsu-1 and Kas-1 [70]. Similar to the previous study, transcript profiling identified vast constitutive differences between accessions, as well as a set of genes exhibiting differential response to relatively mild drought treatment. Another example, although not directly linked to abiotic stress, indicates the power of using large numbers of contrasting lines to identify direct links between observed phenotype and molecular profiles [71]. Rather than using many different accessions, phenotypic variation in plant biomass was captured in a collection of recombinant inbred lines (RILs) obtained from Col-0 × C24 cross. Using multivariate correlation, metabolite profiles obtained for more than 200 RILs, and biomass information was sufficient to identify combinations of metabolites that significantly correlated with plant size. Surprisingly, the number of primary metabolites including energy sources such as sucrose and fumarate was negatively correlated with growth suggesting that in contrast to the former dogma that it is not metabolism that drives growth but it is rather growth that drives metabolism. In a subsequent study, the obtained data were used to successfully overlay QTL for biomass and metabolite concentrations providing list of candidate target genes such as those involved in myo-inositol metabolism [72].

13.4.3
The Use of Tissue and Cell-Specific Analysis

It is obvious that physiological responses to stress vary between the plant organs and the underlying cell types, with some of the effects being restricted to only certain cell types, while other responses are more general. This was elegantly demonstrated to also apply at the molecular level via a transcript profiling study of *Arabidopsis* roots subjected to high salt concentration, which showed that transcriptional stress responses depended highly on the cell type [60]. More specifically, roots were divided into four longitudinal regions corresponding to their developmental fate (meristematic, elongation, and mature) and six different GFP-marker lines were used to capture different

cell types present in the root. Remarkably, only a small proportion of the genes showed significant transcriptional changes in multiple developmental zones and tissue layers, whereas most of the responses were development or tissue specific. When compared to whole-root profiles, many of the specific effects were masked and thus not reported previously. Moreover, the observed changes made logical connections to the observed root phenotypes and physiology. For example, inhibition of root hair outgrowth, which prevents salt uptake into the vasculature, could be linked to downregulation of genes involved in root hair initiation in epidermis [60]. Similar conclusions could be drawn from the consequent work of Skirycz *et al.* (2010) comparing expression and metabolite profiles of growing (fully proliferating and fully expanding) versus mature *Arabidopsis* leaves harvested from plants subjected to prolonged mild osmotic stress [61]. Only very few genes and metabolites responded to stress at all leaf stages, and mature and proliferating leaves were particularly distinct. While the "classical" abscisic acid-mediated stress response was prevalent at the mature stage, ethylene and gibberellin signaling played a more prominent role in the growing leaves, and effector genes were proliferation or expansion specific. These two studies highlight the importance of tissue- or even cell-specific profiling of stress responses in order to understand the molecular basis of stress phenotypes.

13.4.4
The Use of Stress Combinations

In their natural habitats, in contrast to lab conditions, plants are usually subjected to a combination of abiotic stresses that will interact with each other (reviewed by Mittler [73]). For example, drought is often combined with heat, and while drought promotes the closure of stomata, this will lead to an increase in leaf temperature that is not optimal if combined with heat stress. In agreement, the molecular response to the combination of drought and heat stress was found to be distinct from that of plants subjected to drought or heat stress alone. Several hundred transcripts and a number of metabolites specific to the combination could be identified. For instance, plants subjected to heat and drought combination accumulated sugars rather than proline as the major osmoprotectants, as proline aggravated deleterious effects of heat. Analogous findings were also reported for other stress combinations emphasizing the importance of optimizing lab testing conditions in a way that they more resemble field conditions of interest.

13.5
Applications of "Omics" Data in the Improvement of Stress Tolerance

13.5.1
Lead Gene Discovery

"Omics" approaches are powerful tools for hypothesis generation with respect to lead discovery in the context of applied research. In the simplest sense, genes that respond

to a certain abiotic stimulus can be interesting direct or indirect candidate genes to direct approaches for engineering a specific trait including abiotic stress tolerance. This concept was demonstrated even before the adoption of "omics" approaches using the strongly cold-induced transcription factors C-repeat binding factor1 (CBF1), CBF2, and CBF3 [74], also known as dehydration-responsive element binding1b (DREB1b), DREB1c, and DREB1a, respectively [75], to engineer *Arabidopsis* plants with increased freezing tolerance [75, 76]. Subsequently, the CBF genes have been shown to be among those most consistently observed to be cold induced in expression profiling studies (reviewed in Ref. [77]) and they were also genes identified by regression analysis that were significantly correlated with acclimated freezing tolerance [28], validating that "omics" approaches can, in principle, be used to select candidate genes that are likely to give positive abiotic stress phenotypes.

Although a positive phenotypic effect is clearly desired from an applied perspective, negative phenotypes are arguably more informative in the characterization of intrinsic plant stress responses. For example, a broad comparison of transcript profiles obtained from plants exposed to 2 heat stress regimes, which led to different heat acclimation phenotypes, allowed the identification of 8 genes (from approximately 30 tested) where heat acclimation was perturbed in loss-of-function mutants [57]. Besides identifying genes relevant to stress response pathways, such negative loss-of-function phenotypes can also indicate an increased likelihood that the overexpression of those same genes may lead to improved stress tolerance. For example, the double loss-of-function mutant for two hypoxia-responsive ERF genes, HRE1 and HRE2, had increased susceptibility to anoxia, whereas the overexpression of HRE1 increased tolerance [78]. Obviously, it can also be the case that genes identified through "omics" approaches can be negative regulators and thus have a positive phenotype for loss of function. This was the case for the cold-induced cytokinin nuclear response regulator ARR7, where the *arr7* mutant had increased freezing tolerance [79].

As mentioned in the earlier sections, "whole-plant" analyses using "omics" approaches can have a low resolution and potentially miss genes that are essential components of the stress response. The utility of cell- or tissue-specific analyses for the discovery of genes affecting plant abiotic stress tolerance has been investigated only in a few cases, but has yielded promising results. For example, using cell-specific analysis, POPEYE, a bHLH transcription factor, was identified to be induced in root pericyle cells by iron deficiency and to be necessary for iron homeostasis [80]. With respect to tissue-specific differences, the analysis of different leaf stages identified alternative oxidase1a (AOX1a) as being responsive to mild osmotic stress specifically in growing tissues and that its overexpression conferred increased drought tolerance [61]. Another consideration, which has similarly been demonstrated to be useful for gene discovery, is the use of "omics" approaches in combination with exposure to a combination of abiotic stresses. For instance, analysis of the response of *Arabidopsis* seedlings to heat, anoxia, and combined heat and anoxia stress identified heat shock factor A2 (HsfA2) as a candidate gene and that loss-of-function lines were impaired in heat-dependent acclimation to anoxia, while overexpression lines were more tolerant to anoxia [81].

Given that transcriptomics data can be generally useful for gene discovery and there is an increasing body of publicly available data sets, it is perhaps not surprising

that the combined analysis of multiple data sets is gaining increasing attention. The utility of such an approach was demonstrated by Kant *et al*. who performed a meta-analysis of multiple data sets, including their own, for the most consistent stress-responsive genes. After filtering these genes for those annotated with "regulatory" functions (e.g., transcription factors or kinases), they ended up with a large number of candidate genes [82]. Among the first genes they tested, they showed reduced tolerance for a loss-of-function mutant in the clock component genes CCA1/LHY [82] and enhanced stress tolerance in loss-of-function mutants of two RNA helicases named stress-responsive suppressor1 and 2 (STRS) [83].

Metabolomics data and combined analysis of transcripts and metabolites have also proven a useful strategy for directing functional characterization efforts for determining the contributions of key metabolic pathways to plant abiotic stress responses. The observed accumulation of galactinol and raffinose and the concomitant increases in transcripts for galactinol synthase led to the demonstration that its overexpression could increase galactinol and raffinose and confer increased tolerance to drought [84]. Such approaches are not always so clear; for example, although similar increases in galactinol synthase transcripts and galactinol and raffinose are seen during cold acclimation, raffinose was demonstrated to be neither necessary nor sufficient for increased freezing tolerance [85]. On the other hand, other similar approaches investigating cold responses in *Arabidopsis* have been successful. The use of parallel transcript and metabolite profiling identified maltose and beta-amylase transcripts as being strongly induced, and the RNAi downregulation of beta-amylase reduced cold acclimation [86]. Similarly, the cold induction of polyamines and their biosynthetic enzymes directed efforts to characterize their role in plant cold acclimation. Here, the *adc1* and *adc2* loss-of-function biosynthetic mutants had reduced freezing tolerance, although in this case the effect seemed to be at least partially indirect due to interaction with ABA signaling pathway [87].

Most of these examples are based on candidate genes that were identified using data analysis approaches essentially as described in detail in Section 13.3.2. It was already noted that correlation or network approaches and more advanced software allowing data integration and visualization are becoming more important for hypothesis generation. In the context of plant environmental responses, an interesting approach was recently described in which the authors used network-based approaches including coexpression analysis to identify ELF3 as the best candidate gene for a shade-avoidance QTL they had identified [88]. In the future, it is likely that such approaches will be increasingly used, and become sophisticated, for the identification of lead genes or gene combinations for applied discovery research programs.

13.5.2
Promoter Discovery

The need for inducible or specific promoters can be a key tool in plant biotechnology and their need will increase as more complex and refined approaches are utilized in trait engineering. The potential for such approaches has been demonstrated for engineering drought tolerance via stress-inducible expression of a transcription factor [89] or for improving salt tolerance via specific expression of a sodium transporter in the root

stele [90]. Although the promoters in these two examples were not identified via "omics" approaches, specific experiments or publicly available microarray data can be a useful tool with which to identify genes from which to isolate such tissue- or cell-specific promoters. This approach is elegantly demonstrated by Yang *et al.*, who used a combination of public microarray data and their own experiments to identify genes that were highly expressed in stomata guard cells but lowly expressed in leaves, from which they isolated a specific promoter [91]. Several other cell-specific promoters isolated either individually or from GFP enhancer-trap screens [92] are available and have been used to characterize cell-specific responses to stress either at the transcriptome [60] or at the translatome [19] level. Although there are at present few examples of engineered cell-specific stress tolerance, it is likely that the availability of these lines and the genes identified via such cell-specific "omics" approaches will prove useful for future efforts to engineer abiotic stress tolerance via cell-specific approaches. Equally, the use of stress-specific promoters will also be of high value, and although the potential for cell × environment-specific promoters is clear, the obvious complexity is daunting and will likely require several years before their application becomes routine.

13.5.3
Mode-of-Action Characterization

The other main use of "omics" approaches is for the functional characterization of candidate loss- or gain-of-function lines with altered phenotype. This is somewhat analogous to the characterization of mutants described in Section 13.4.2; however, it is distinct as in this case that it is specifically directed at understanding the mode of action underlying candidate genes/events already being examined in an applied context. In *Arabidopsis*, detailed characterization using "omics" approaches is mainly used to drive additional rounds of discovery research by directly or indirectly providing additional candidate genes. One such example is the characterization of *Arabidopsis* plants with RNAi downregulation of poly-ADP-ribose polymerase 2 (PARP2), which provided new insight into how stress tolerance may be conferred through the activation of a known stress signaling pathway. Transcript profiling showed that ABA-responsive genes were upregulated and directed subsequent analysis demonstrating that ABA levels were increased [93]. However, such analysis is not always informative as either few genes may be altered or those that change may not provide any insight into the mode of action, particularly if it is previously unknown or is beyond the cell, tissue, or time resolution investigated – this proved to be the case in the characterization of the transcriptome in *Arabidopsis* lines overexpressing nuclear factor B subunits [94], where no particular molecular basis could be observed.

13.6
Conclusions and Prospects

The application of "omics" approaches in plant sciences in general and in abiotic stress research in particular has become increasingly routine over the past decade.

Although they offer unique coverage of molecular changes, these approaches are useful provided good experimental design is applied, data are appropriately analyzed, and the underlying technical limitations and assumptions are kept in mind when biologically interpreting results. Despite these caveats, their use has greatly accelerated the characterization of plant abiotic stress responses and provided a wealth of data for hypothesis generation. Their utility has been demonstrated in the context of applied research for candidate gene and promoter discovery and the characterization of plants in which improved abiotic stress tolerance has been engineered. However, given the complexity of plant stress responses it can be anticipated that we are only at an early phase in terms of engineering plants with improved stress tolerance when the complexities such as cell and tissue specificity, stress combinations, and interaction between them are considered. There is no doubt that considering the advances already made in these aspects, we will see in the future increasingly elegant solutions to improve plant stress tolerance, often driven by expert knowledge on plant physiology. However, given the potential search space that is available in this potential matrix, it is likely that computational approaches, based on the increasing availability of "omics" data, will become ever more important in order to generate hypotheses and predict which combinations to test.

References

1 The Arabidopsis Genome Initiative (2000) *Nature* **408**, 796–815.
2 Metzker, M.L. (2010) *Nat. Rev. Genet.*, **11**, 31–46.
3 Schneeberger, K., Ossowski, S., Lanz, C., Juul, T., Petersen, A.H., Nielsen, K.L., Jørgensen, J., Weigel, D., and Andersen, S.U. (2009) *Nat. Methods*, **6**, 550–551.
4 Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., and Zimmermann, P. (2008) *Adv. Bioinformatics*, **2008**, 420747.
5 Sclep, G., Allemeersch, J., Liechti, R., De Meyer, B., Beynon, J., Bhalerao, R., Moreau, Y., Nietfeld, W., Renou, J., Reymond, P., Kuiper, M.T., and Hilson, P. (2007) *BMC Bioinformatics*, **8**, 400.
6 Rehrauer, H., Aquino, C., Gruissem, W., Henz, S.R., Hilson, P., Laubinger, S., Naouar, N., Patrignani, A., Rombauts, S., Shu, H., Van de Peer, Y., Vuylsteke, M., Weigel, D., Zeller, G., and Hennig, L. (2010) *Plant Physiol.*, **152**, 487–499.
7 Czechowski, T., Bari, R.P., Stitt, M., Scheible, W., and Udvardi, M.K. (2004) *Plant J.*, **38**, 366–379.
8 Geiss, G.K., Bumgarner, R.E., Birditt, B., Dahl, T., Dowidar, N., Dunaway, D.L., Fell, H.P., Ferree, S., George, R.D., Grogan, T., James, J.J., Maysuria, M., Mitton, J.D., Oliveri, P., Osborn, J.L., Peng, T., Ratcliffe, A.L., Webster, P.J., Davidson, E.H., Hood, L., and Dimitrov, K. (2008) *Nat. Biotechnol.*, **26** 317–325.
9 Lipson, D., Raz, T., Kieu, A., Jones, D.R., Giladi, E., Thayer, E., Thompson, J.F., Letovsky, S., Milos, P., and Causey, M. (2009) *Nat. Biotechnol.*, **27**, 652–658.
10 Wienkoop, S., Baginsky, S., and Weckwerth, W. (2010) *J. Proteomics*. doi: 10.1016/j.jprot.2010.07.012
11 Gouw, J.W., Krijgsveld, J., and Heck, A.J.R. (2010) *Mol. Cell Proteomics*, **9**, 11–24.
12 Saito, K. and Matsuda, F. (2010) *Annu. Rev. Plant Biol.*, **61**, 463–489.
13 Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., and Fernie, A.R. (2006) *Nat. Protoc.*, **1**, 387–396.
14 Giavalisco, P., Köhl, K., Hummel, J., Seiwert, B., and Willmitzer, L. (2009) *Anal. Chem.*, **81**, 6546–6551.

15 Chinnusamy, V. and Zhu, J. (2009) *Curr. Opin. Plant Biol.*, **12**, 133–139.

16 Lister, R., O'Malley, R.C., Tonti-Filippini, J., Gregory, B.D., Berry, C.C., Millar, A.H., and Ecker, J.R. (2008) *Cell*, **133**, 523–536.

17 Villar, C.B.R. and Köhler, C. (2010) *Methods Mol. Biol.*, **655**, 401–411.

18 Kaufmann, K., Muiño, J.M., Østera7pt°.61pts, M., Farinelli, L., Krajewski, P., and Angenent, G.C. (2010) *Nat. Protoc.*, **5**, 457–472.

19 Mustroph, A., Zanetti, M.E., Jang, C.J.H., Holtan, H.E., Repetti, P.P., Galbraith, D.W., Girke, T., and Bailey-Serres, J. (2009) *Proc. Natl. Acad. Sci. USA*, **106**, 18843–18848.

20 Narsai, R., Howell, K.A., Millar, A.H., O'Toole, N., Small, I., and Whelan, J. (2007) *Plant Cell*, **19**, 3418–3436.

21 Van Leene, J., Hollunder, J., Eeckhout, D., Persiau, G., Van De Slijke, E., Stals, H., Van Isterdael, G., Verkest, A., Neirynck, S., Buffel, Y., De Bodt, S., Maere, S., Laukens, K., Pharazyn, A., Ferreira, P.C.G., Eloy, N., Renne, C., Meyer, C., Faure, J., Steinbrenner, J., Beynon, J., Larkin, J.C., Van de Peer, Y., Hilson, P., Kuiper, M., De Veylder, L., Van Onckelen, H., Inze, D., Witters, E., and De Jaeger, G. (2010) *Mol. Syst. Biol.*, **6**. doi: 10.1038/msb.2010.53

22 Salt, D.E., Baxter, I., and Lahner, B. (2008) *Annu. Rev. Plant Biol.*, **59**, 709–733.

23 Nettleton, D. (2006) *Plant Cell*, **18**, 2112–2121.

24 van de Peppel, J., Kemmeren, P., van Bakel, H., Radonjic, M., van Leenen, D., and Holstege, F.C. (2003) *EMBO Rep.*, **4**, 387–393.

25 Hannah, M.A., Redestig, H., Leisse, A., and Willmitzer, L. (2008) *Nat. Biotechnol.*, **26**, 741–742.

26 Allison, D.B., Cui, X., Page, G.P., and Sabripour, M. (2006) *Nat. Rev. Genet.*, **7**, 55–65.

27 Ting, L., Cowley, M.J., Hoon, S.L., Guilhaus, M., Raftery, M.J., and Cavicchioli, R. (2009) *Mol. Cell Proteomics*, **8**, 2227–2242.

28 Hannah, M.A., Wiese, D., Freund, S., Fiehn, O., Heyer, A.G., and Hincha, D.K. (2006) *Plant Physiol.*, **142**, 98–112.

29 Redestig, H., Weicht, D., Selbig, J., and Hannah, M.A. (2007) *BMC Bioinformatics*, **8**, 454.

30 Michoel, T., Maere, S., Bonnet, E., Joshi, A., Saeys, Y., Van den Bulcke, T., Van Leemput, K., van Remortel, P., Kuiper, M., Marchal, K., and Van de Peer, Y. (2007) *BMC Bioinformatics*, **8**, S5.

31 Hirai, M.Y., Sugiyama, K., Sawada, Y., Tohge, T., Obayashi, T., Suzuki, A., Araki, R., Sakurai, N., Suzuki, H., Aoki, K., Goda, H., Nishizawa, O.I., Shibata, D., and Saito, K. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 6478–6483.

32 Tohge, T., Nishiyama, Y., Hirai, M.Y., Yano, M., Nakajima, J., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D.B., Kitayama, M., Noji, M., Yamazaki, M., and Saito, K. (2005) *Plant J.*, **42**, 218–235.

33 Hannah, M.A., Caldana, C., Steinhauser, D., Balbo, I., Fernie, A.R., and Willmitzer, L. (2010) *Plant Physiol.*, **152**, 2120–2129.

34 Maere, S., Heymans, K., and Kuiper, M. (2005) *Bioinformatics*, **21**, 3448–3449.

35 Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., Selbig, J., Müller, L.A., Rhee, S.Y., and Stitt, M. (2004) *Plant J.*, **37**, 914–939.

36 Poree, F., Fernie, A.R., Stitt, M., Usadel, B., Nagel, A., Steinhauser, D., Gibon, Y., Bläsing, O.E., Redestig, H., Sreenivasulu, N., Krall, L., and Hannah, M.A. (2006) *BMC Bioinformatics*, **7**, 535.

37 Tokimatsu, T., Sakurai, N., Suzuki, H., Ohta, H., Nishitani, K., Koyama, T., Umezawa, T., Misawa, N., Saito, K., and Shibata, D. (2005) *Plant Physiol.*, **138**, 1289–1300.

38 Zhang, P., Foerster, H., Tissier, C.P., Mueller, L., Paley, S., Karp, P.D., and Rhee, S.Y. (2005) *Plant Physiol.*, **138**, 27–37.

39 Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., and Provart, N.J. (2007) *PLoS ONE*, **2**, e718.

40 Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004) *Plant Physiol.*, **136**, 2621–2632.

41 Usadel, B., Obayashi, T., Mutwil, M., Giorgi, F.M., Bassel, G.W., Tanimoto, M., Chow, A., Steinhauser, D., Persson, S., and Provart, N.J. (2009) *Plant Cell Environ.*, **32**, 1633–1651.

42 Brady, S.M. and Provart, N.J. (2009) *Plant Cell*, **21**, 1034–1051.

43 Katari, M.S., Nowicki, S.D., Aceituno, F.F., Nero, D., Kelfer, J., Thompson, L.P., Cabello, J.M., Davidson, R.S., Goldberg, A.P., Shasha, D.E., Coruzzi, G.M., and

Gutierrez, R.A. (2010) *Plant Physiol.*, **152**, 500–515.
44 Verslues, P.E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., and Zhu, J. (2006) *Plant J.*, **45**, 523–539.
45 Mittler, R. and Blumwald, E. (2010) *Annu. Rev. Plant Biol.*, **61**, 443–462.
46 Hirayama, T. and Shinozaki, K. (2010) *Plant J.*, **61**, 1041–1052.
47 Kreps, J.A., Wu, Y., Chang, H., Zhu, T., Wang, X., and Harper, J.F. (2002) *Plant Physiol.*, **130**, 2129–2141.
48 Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., and Shinozaki, K. (2002) *Plant J.*, **31**, 279–292.
49 Matsui, A., Ishida, J., Morosawa, T., Mochizuki, Y., Kaminuma, E., Endo, T.A., Okamoto, M., Nambara, E., Nakajima, M., Kawashima, M., Satou, M., Kim, J., Kobayashi, N., Toyoda, T., Shinozaki, K., and Seki, M. (2008) *Plant Cell Physiol.*, **49**, 1135–1149.
50 Zeller, G., Henz, S.R., Widmer, C.K., Sachsenberg, T., Rätsch, G., Weigel, D., and Laubinger, S. (2009) *Plant J.*, **58**, 1068–1082.
51 Seki, M., Umezawa, T., Urano, K., and Shinozaki, K. (2007) *Curr. Opin. Plant Biol.*, **10**, 296–302.
52 Sweetlove, L.J., Heazlewood, J.L., Herald, V., Holtzapffel, R., Day, D.A., Leaver, C.J., and Millar, A.H. (2002) *Plant J.*, **32**, 891–904.
53 Ndimba, B.K., Chivasa, S., Simon, W.J., and Slabas, A.R. (2005) *Proteomics*, **5**, 4185–4196.
54 Koussevitzky, S., Suzuki, N., Huntington, S., Armijo, L., Sha, W., Cortes, D., Shulaev, V., and Mittler, R. (2008) *J. Biol. Chem.*, **283**, 34197–34203.
55 Pang, Q., Chen, S., Dai, S., Chen, Y., Wang, Y., and Yan, X. (2010) *J. Proteome Res.*, **9**, 2584–2599.
56 Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., and Harter, K. (2007) *Plant J.*, **50**, 347–363.
57 Larkindale, J. and Vierling, E. (2008) *Plant Physiol.*, **146**, 748–761.
58 Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., and Mittler, R. (2004) *Plant Physiol.*, **134**, 1683–1696.
59 Bieniawska, Z., Espinoza, C., Schlereth, A., Sulpice, R., Hincha, D.K., and Hannah, M.A. (2008) *Plant Physiol.*, **147**, 263–279.
60 Dinneny, J.R., Long, T.A., Wang, J.Y., Jung, J.W., Mace, D., Pointer, S., Barron, C., Brady, S.M., Schiefelbein, J., and Benfey, P.N. (2008) *Science*, **320**, 942–945.
61 Skirycz, A., De Bodt, S., Obata, T., De Clercq, I., Claeys, H., De Rycke, R., Andriankaja, M., Van Aken, O., Van Breusegem, F., Fernie, A.R., and Inze, D. (2010) *Plant Physiol.*, **152**, 226–244.
62 Wilkins, O., Bräutigam, K., and Campbell, M.M. (2010) *Plant J.* doi: 10.1111/j.1365-313X.2010.04274.x
63 Hummel, I., Pantin, F., Sulpice, R., Piques, M., Rolland, G., Dauzat, M., Christophe, A., Pervent, M., Bouteille, M., Stitt, M., Gibon, Y., and Muller, B. (2010) *Plant Physiol.*, **154**, 357–372.
64 Kim, J.K., Bamba, T., Harada, K., Fukusaki, E., and Kobayashi, A. (2007) *J. Exp. Bot.*, **58**, 415–424.
65 Urano, K., Maruyama, K., Ogata, Y., Morishita, Y., Takeda, M., Sakurai, N., Suzuki, H., Saito, K., Shibata, D., Kobayashi, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2009) *The Plant J.*, **57**, 1065–1078.
66 Maruyama, K., Sakuma, Y., Kasuga, M., Ito, Y., Seki, M., Goda, H., Shimada, Y., Yoshida, S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004) *Plant J.*, **38**, 982–993.
67 Sakuma, Y., Maruyama, K., Osakabe, Y., Qin, F., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) *Plant Cell*, **18**, 1292–1309.
68 Vogel, J.T., Zarka, D.G., Van Buskirk, H.A., Fowler, S.G., and Thomashow, M.F. (2005) *Plant J.*, **41**, 195–211.
69 Lefebvre, V., Kiani, S.P., and Durand-Tardif, M. (2009) *Int. J. Mol. Sci.*, **10**, 3547–3582.
70 Juenger, T.E., Sen, S., Bray, E., Stahl, E., Wayne, T., McKay, J., and Richards, J.H. (2010) *Plant Cell Environ.*, **33**, 1268–1284.
71 Meyer, R.C., Steinfath, M., Lisec, J., Becher, M., Witucka-Wall, H., Törjék, O., Fiehn, O., Eckardt, Ä., Willmitzer, L., Selbig, J., and Altmann, T. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 4759–4764.

72 Lisec, J., Steinfath, M., Meyer, R.C., Selbig, J., Melchinger, A.E., Willmitzer, L., and Altmann, T. (2009) *Plant J.*, **59**, 777–788.

73 Mittler, R. (2006) *Trends Plant Sci.*, **11**, 15–19.

74 Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M., and Thomashow, M.F. (1998) *Plant J.*, **16**, 433–442.

75 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) *Plant Cell*, **10**, 1391–1406.

76 Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998) *Science*, **280**, 104–106.

77 Hannah, M.A., Heyer, A.G., and Hincha, D.K. (2005) *PLoS Genet.*, **1**, e26.

78 Licausi, F., van Dongen, J.T., Giuntoli, B., Novi, G., Santaniello, A., Geigenberger, P., and Perata, P. (2010) *Plant J.*, **62**, 302–315.

79 Jeon, J., Kim, N.Y., Kim, S., Kang, N.Y., Novák, O., Ku, S., Cho, C., Lee, D.J., Lee, E., Strnad, M., and Kim, J. (2010) *J. Biol. Chem.*, **285**, 23371–23386.

80 Long, T.A., Tsukagoshi, H., Busch, W., Lahner, B., Salt, D.E., and Benfey, P.N. (2010) *Plant Cell*, **22**, 2219–2236.

81 Banti, V., Loreti, E., Novi, G., Santaniello, A., Alpi, A., and Perata, P. (2008) *Plant Cell Environ.*, **31**, 1029–1037.

82 Kant, P., Gordon, M., Kant, S., Zolla, G., Davydov, O., Heimer, Y.M., Chalifa-Caspi, V., Shaked, R., and Barak, S. (2008) *Plant Cell Environ.*, **31**, 697–714.

83 Kant, P., Kant, S., Gordon, M., Shaked, R., and Barak, S. (2007) *Plant Physiol.*, **145**, 814–830.

84 Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2002) *Plant J.*, **29**, 417–426.

85 Zuther, E., Büchel, K., Hundertmark, M., Stitt, M., Hincha, D.K., and Heyer, A.G. (2004) *FEBS Lett.*, **576**, 169–173.

86 Kaplan, F. and Guy, C.L. (2005) *Plant J.*, **44**, 730–743.

87 Cuevas, J.C., Lopez-Cobollo, R., Alcazar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., and Ferrando, A. (2008) *Plant Physiol.*, **148**, 1094.

88 Jiménez-Gómez, J.M., Wallace, A.D., and Maloof, J.N. (2010) *PLoS Genet.*, **6**. doi: 10.1371/journal.pgen.1001100

89 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) *Nat. Biotechnol.*, **17**, 287–291.

90 Moller, I.S., Gilliham, M., Jha, D., Mayo, G.M., Roy, S.J., Coates, J.C., Haseloff, J., and Tester, M. (2009) *Plant Cell*, **21**, 2163–2178.

91 Yang, Y., Costa, A., Leonhardt, N., Siegel, R., and Schroeder, J. (2008) *Plant Methods*, **4**, 6.

92 Gardner, M.J., Baker, A.J., Assie, J., Poethig, R.S., Haseloff, J.P., and Webb, A.A.R. (2009) *J. Exp. Bot.*, **60**, 213–226.

93 Vanderauwera, S., De Block, M., Van de Steene, N., Van De Cotte, B., Metzlaff, M., and Van Breusegem, F. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 15150.

94 Donnarummo, M.G., Nelson, D.E., Repetti, P.P., Adams, T.R., Creelman, R.A., Wu, J., Warner, D.C., Anstrom, D.C., Bensen, R.J., and Castiglioni, P.P. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 16450.

14
Functional Genomics and Computational Biology Tools for Gene Discovery for Abiotic Stress Tolerance

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Unraveling the molecular details of plant response and defense against abiotic stress factors such as drought, salt, and temperature extremes is a crucial and challenging issue in plant research. Functional genomics and computational biology have enhanced the pace of molecular dissection of abiotic stress response mechanisms. In the past two decades, significant progress has been made in identification of genes involved in abiotic stress responses in model plants *Arabidopsis* and rice through forward and reverse genetic analyses. Besides, QTL analysis is a powerful complementary technology with functional genomics to discover and isolate the genes of agronomic importance. Several QTL associated with abiotic stress responses of plants have been mapped. The availability of complete genome sequence of important model plants, namely, *Arabidopsis* and rice, QTL databases, and mapping tools facilitates genomics-based strategies for gene discovery, coupled with high-throughput techniques, for abiotic stress tolerance. Fine mapping of these QTL will help the identification of major genes and development of tightly linked molecular markers that can be employed to genetically improve crops through genetic engineering and marker-assisted selection (MAS) breeding.

14.1
Introduction

Burgeoning population imposes huge pressure on agriculture to produce more food from shrinking land and other natural resources. Hence, development of high-yield and input use-efficient crops is necessary to meet the growing food, fiber, fodder, and fuel demand. Unfortunately, plants are constantly exposed to a variety of environmental factors such as biotic and abiotic stresses, causing deleterious effects on growth, development, and thus productivity of crops [1]. Biotic stress includes insect and pathogen infection, while abiotic stresses include extremes of temperature (low/high), water availability (drought/water logging), light (low/high), and minerals

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(deficiency and toxicity). To overcome the yield losses due to biotic and abiotic stresses, plants have evolved different strategies to avoid or manage with the stress conditions. Higher plants have evolved multiple interconnected strategies that enable them to survive under stress conditions. Plants exhibit various responses to these stresses at the molecular, cellular, and whole-plant levels [2–7]. Plants respond to stresses partly by activating the expression of stress-responsive genes that leads to adaptation at morphophysiological and cellular levels. Besides molecular genetic approaches, quantitative trait loci (QTL) mapping is an important approach for genetic dissection of complex agronomic traits for plants and thus key to the improvement of crop yield. QTL cloning by high-resolution mapping enables identification of gene(s) underlying the QTL [8]. Stress-responsive genes/expressed sequence tags (ESTs) provide a valuable source for development of gene-based markers for fine mapping of QTL. For instance, ~200 SNP markers have been developed and mapped in barley, on the basis of their differential transcription response to abiotic stresses [9]. The availability of complete genome sequence of the model plant *Arabidopsis* [10] and rice [11, 12] spp. *indica* [13] and *japonica* [14] was the first step toward the understanding the genetic complexity of abiotic stress responses. These genome sequencing programs have been complemented by rapid gene discovery from large-scale EST sequencing in *Arabidopsis* [15–18] and rice [19, 20]. In important crops that have not been sequenced or their sequencing is in progress, large-scale EST sequencing initiatives provide a cost-effective source of obtaining information about the transcribed genes of individual species [21]. Complete genome sequence and ESTs provide the necessary prerequisite for high-throughput, large-scale gene expression profiling. EST resources generated by large-scale transcript profiling reveal differential expression patterns that can often provide clues to gene function [22]. The publicly available ESTs are growing rapidly and can be obtained from NCBI-dbEST (http://www.ncbi.nlm.nih.gov/dbEST/). The recently emerged "omics" technologies (e.g., genomics, proteomics, transcriptomics, and metabolomics) have enhanced the power of deciphering the functional information at the gene level in a shorter period of time [23]. Gene characterization by forward and reverse genetics under stress conditions has significantly boosted transgenic development. Functional genomics and computational biology tools (e.g., QTL mapping and trait viewer) provide precious support to decipher genetic determinants of abiotic stress tolerance, accelerating the ongoing research on QTL/gene discovery. The objective of QTL mapping is to determine candidate genes that are responsible for variation in complex, quantitative traits [24]. They also open up new opportunities to improve stress tolerance by incorporating genes involved in stress protection from any source into agriculturally important crop traits. The availability of genomic information provides systematic candidate gene discovery with the help of various bioinformatics-based QTL mapping tools. Recently, three QTL genes have been successfully identified as candidate genes in rice, which help to understand plants' response to abiotic stresses [25–27]. Understanding the mechanisms of regulation of stress-responsive genes will help us to breed or engineer stress-tolerant crop plants. For a sustainable development of agriculture, future crops should have abiotic stress-resistant traits.

14.2
Gene Discovery in Model Organism

Agriculturally important traits such as yield, quality, and disease resistance are controlled by many genes and are known as quantitative traits (also known as "polygenic," "multifactorial," or "complex" traits). The regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci. DNA (or molecular) markers are used to construct linkage maps, which are useful for gene and QTL mapping [28]. The process of QTL analysis – to identify genomic regions associated with traits – is known as QTL mapping (also called "genetic," "gene," or "genome" mapping) [28–30]. Mapping agronomically important genes can provide useful information to plant breeders. The QTL approach will facilitate gene discovery and will focus on the mechanisms that allow plants to adapt to harsh environments. As plants experience stress at different stages of growth and development, it would be useful if the genes/QTL are identified with a specific role at a given growth stage in model plant species. Discovery of genes controlling economically important traits can be used for applications in marker-assisted selection (MAS) breeding and improving crops using transgenic approaches. Gene identification from model organisms was successfully used to demonstrate their possible use in improving stress tolerance of transgenic crop plants [31–33]. Past efforts to improve plant tolerance to drought, high salinity, and low temperature through breeding and genetic engineering have had limited success owing to the genetic complexity of stress responses. The discovery of novel genes and functional variation in their roles in stress adaptation is fundamental to effective engineering strategies to impart plants greater stress tolerance. A large number of genes involved in abiotic stress responses have been cloned and characterized in recent years. Rice and *Arabidopsis* model plants have played an important role in understanding abiotic stress tolerance mechanisms [34]. Identification of abiotic stress-responsive *cis*-regulatory elements helped unravel stress signaling mechanisms, for example, dehydration-responsive element (DRE)/C-repeat (CRT) (A/GCCGAC) binding transcription factors and DRE binding protein (DREB)/C-repeat binding factor (CBF) [35, 36]. The future of crop improvement through genetic improvements can be accomplished in three major phases: First, identification of loci involved in the environmental stress tolerance at reproductive stage. Forward and reverse genetics in model plants, such as *Arabidopsis* and rice, and their tolerant relatives will play a key role in this process in years to come. Second, the appropriate alleles for the major loci will need to be identified. Association (linkage disequilibrium) mapping with genome-wide association studies will help identify useful alleles. Wild relatives of each crop will be essential for this. Finally, the translational research to develop stress-tolerant genotype by MAS and/or transgenic approach is necessary. The completion of several plant genome sequencing projects has provided public resources of genomic data for forward and reverse genetics, comparative genomics, *in silico* predictions, QTL mapping, and cloning. Some genome sequence and annotation databases for plant species are listed in Table 14.1.

Table 14.1 Sequence and annotation databases for plant genome.

Plant genome database	URL
Arabidopsis (PlantGDB)	http://www.plantgdb.org/AtGDB/
Arabidopsis (TAIR)	http://www.arabidopsis.org/
Arabidopsis (NCBI)	ftp://ftp.ncbi.nih.gov/genomes/Arabidopsis_thaliana/
Rice (PlantGDB)	http://www.plantgdb.org/OsGDB/
Rice (TIGR)	http://rice.plantbiology.msu.edu/
Rice (NCBI)	ftp://ftp.ncbi.nih.gov/genomes/Oryza_sativa/
Soybean (PlantGDB)	http://www.plantgdb.org/GmGDB/
Soybean (NCBI)	ftp://ftp.ncbi.nih.gov/genomes/PLANTS/Glycine_max/
Sorghum (PlantGDB)	http://www.plantgdb.org/SbGDB/
Sorghum (NCBI)	ftp://ftp.ncbi.nih.gov/genomes/PLANTS/Sorghum_bicolor/
Maize (PlantGDB)	http://www.plantgdb.org/ZmGDB/
Maize (TIGR)	http://maize.jcvi.org/
Maize (NCBI)	ftp://ftp.ncbi.nih.gov/genomes/PLANTS/Zea_mays/
Wine grapes (PlantGDB)	http://www.plantgdb.org/VvGDB/
Wine grapes (NRCPB)	ftp://ftp.ncbi.nih.gov/genomes/Vitis_vinifera/
Medicago (PlantGDB)	http://www.plantgdb.org/MtGDB/
Medicago (TIGR)	http://www.jcvi.org/cgi-bin/medicago/overview.cgi
Medicago (NCBI)	ftp://ftp.ncbi.nih.gov/genomes/PLANTS/Medicago_truncatula/
Wheat (PlantGDB)	http://www.plantgdb.org/TaGDB/
Wheat (TIGR)	http://blast.jcvi.org/euk-blast/index.cgi?project=tae1
Wheat (NCBI)	ftp://ftp.ncbi.nih.gov/genomes/PLANTS/Triticum_aestivum/
Tomato (PlantGDB)	http://www.plantgdb.org/SlGDB/
Tomato (NCBI)	ftp://ftp.ncbi.nih.gov/genomes/PLANTS/Solanum_lycopersicum/
EST Database	http://compbio.dfci.harvard.edu/tgi/plant.html
dbEST (NCBI)	http://www.ncbi.nlm.nih.gov/dbEST/

14.3 High-Throughput Gene Expression Analysis

The availability of complete genome sequence of *Arabidopsis thaliana* and *Oryza sativa* model plants and other important crops has provided sufficient genomic information to perform high-throughput genome-wide functional analysis. Functional annotation and measurement of expression level of genes under various abiotic stresses are accomplished by various molecular biology techniques [37]. cDNA-AFLP (cDNA-amplified fragment length polymorphism), MPSS (massively parallel signature sequencing), and especially SAGE (serial analysis of gene expression) have been successfully used to quantify transcript abundance for different kinds of tissues or developmental stages in higher plants [38–43]. However, these techniques are time consuming and labor-intensive. High-throughput gene expression measurements have the potential to provide clues to many important biological questions involving stress response and tolerance [37]. Microarray and real-time polymerase chain reaction (RT-PCR) are

powerful functional genomics tools widely used in gene expression analysis in plants [44–51]. Microarray experiments provide significant information about stress-related genes to improve biotic and abiotic stress tolerance in plants. SAGE is another useful technique that allows rapid and detailed analysis of thousands of transcripts. The important fact about SAGE is that this technique does not require a preexisting clone; thus, it can be used to identify and quantitate both new genes and known genes [52–54]. Digital gene expression (DGE) is another approach to study transcriptome of plant crops taking less time and in a cost-effective manner [55–59]. Over the past 5 years, a new technique known as tiling array is being widely used and has become a powerful tool for the whole-genome transcriptome analysis. Tiling array technology is derived from microarray technology. In tiling array, whole genome is tiled on the chip that allows identification of noncoding RNAs. mRNA processing (intron retention/alternate splicing) is useful for ChIP-Chip to identify DNA–protein interaction. The recently developed rapid genome sequencing technology, the next-generation sequencing (NGS), has changed the way a biological problem is studied. NGS-based transcriptome analysis is superior to other available techniques since sequencing-based method is digital, highly accurate, and easy-to-perform and is capable of identifying allele-specific expression. ChIP-Chip (chromatin immunoprecipitation-on-chip), also known as location analysis (LA), is a high-throughput genome-wide identification and analysis of DNA fragments that are bound by specific proteins such as histones and transcriptional factors [60–67]. ChIP is a well-established procedure to investigate interactions between proteins and DNA. This strategy may be used to annotate functional elements, such as promoters, enhancers, repressor elements, and insulators, in genomes by mapping the locations of protein markers associated with these sites. ChIP-Sequencing, also known as ChIP-Seq, is used to analyze protein interactions with DNA. ChIP-Seq combines chromatin immunoprecipitation (ChIP) with massively parallel DNA sequencing to identify the cistrome of DNA-associated proteins. It can be used to precisely map global binding sites for any protein of interest. Chip-Seq is used primarily to determine how transcription factors and other chromatin-associated proteins influence phenotype-affecting mechanisms. Determining how proteins interact with DNA to regulate gene expression is essential for fully understanding many biological processes and disease states. CLIP-Seq [68], also called RIP-Seq [69] or HITS-CLIP [70], is a method in molecular biology used for finding which RNA species interact with a particular RNA binding protein or an RNA [71]. It employs cross-linking between the RNA and the protein, followed by immunoprecipitation with antibodies for the protein, fragmentation, high-throughput sequencing, and bioinformatics. Recently, CLIP-Seq has been applied to decode microRNA–target interaction maps [72–74]. The application of CLIP-Seq methods has significantly reduced the rate of false-positive predictions of miRNA binding sites and has also reduced the size of the search space for miRNA target sites [72, 73]. Frequently used techniques for gene expression analysis are listed in Table 14.2.

Table 14.2 Frequently used techniques for gene expression analysis in plants.

Techniques	Application	References
Microarrays	Microarray analysis has enabled the measurement of thousands of genes in a single RNA sample	[75]
Tiling arrays	Tiling arrays are an improved technology of microarray chips and basically used for the whole-genome transcriptome analysis	
ChIP-Chip (or ChIP-on-chip)	ChIP is a well-established procedure for identification and analysis of DNA fragments that are bound by specific proteins such as histones and transcriptional factors	
ChIP-Seq	ChIP-Seq is a powerful method to identify genome-wide DNA binding sites for proteins of interest	
CLIP-Seq (RIP-Seq or HITS-CLIP)	CLIP-Seq is a method in molecular biology, used to find which RNA species interact with a particular RNA binding protein or RNA	[68–71]
Real-time PCR	PCR is a method that allows exponential amplification of short DNA sequences (usually 100–600 bases) within a longer double-stranded DNA molecule	[76]
cDNA-AFLP	cDNA-amplified fragment length polymorphism is a PCR-based method that starts with cDNA synthesis from total or mRNA using random hexamers as primers	[77]
SAGE	SAGE is an experimental technique that allows a rapid and detailed analysis of thousands of transcripts	[52, 54, 78]
MPSS	Massively parallel signature sequencing is a sequencing-based technology that uses a unique method to quantify gene expression level, generating millions of short-sequence tags per library	[79]
EST	Analysis of expression sequence tags is an efficient way to obtain information about gene expression and coding sequences of uncharacterized genomes. It also gives us clue about transcripts coming from different tissues, cell types, and developmental stages	

14.4 Computational Resources: Databases and Software

Rapid genome sequencing and development of high-throughput "omics" technologies such as genomics, transcriptomics, proteomics, and metabolomics have generated a huge amount of biological data. To fully understand biological processes, researchers require to process and analyze the huge volume of data that requires high computational power. Bioinformatics is increasingly becoming an essential tool for many biological areas including systems biology [80] and plays an important role in processing and extracting biologically relevant information from this vast data. Leading bioinformatics companies are developing software to allow research scientists

to integrate their diverse data and tools under common Graphical User Interfaces (GUIs). Scientists from various organizations are continually submitting biological information in databases, which significantly contributes toward the availability of the same data sets for a wide group of researchers and thus permit rapid analysis of data. Recently, new genomic resources (e.g., QlicRice, Gramene-QTL, etc.) and tools (QTL Cartographer, MapCharts, etc.), facilitating QTL mapping and cloning, have been developed and are maintained by various laboratories. Identification of the responsible genes and their allelic variation underlying, or associated with, phenotypic trait is desired for an effective marker-assisted breeding to enhance tolerance to abiotic stress. Some handy databases and tools useful in stress biology, QTL mapping, and plant genomics are listed in Tables 14.3–14.5.

Table 14.3 Databases dedicated to abiotic stress in plants.

Database	Description	Web	Reference
QlicRice	Platform for abiotic stress-responsive QTL in rice. Database also provides information on gene ontology, biochemical pathway, and 3D structure of proteins related to abiotic stress	http://202.141.12.200/	
PLANT STRESS	Dedicated to plant environmental stress in agriculture and biology	http://www.plantstress.com/	
STIFDB (Stress Responsive Transcription Factor Database)	Collection of abiotic stress responsive genes in *A. thaliana*, including transcription factor binding sites in their promoters. Abiotic stress (drought, cold, salinity, high light, heat, etc.) responsive transcription factors have been included in this database	http://caps.ncbs.res.in/stifdb/index.html	[81]
PSGD (Plant Stress Gene Database)	Database contains ~259 biotic and abiotic stress-related genes of 11 species (*A. thaliana*, *Arachis hypogaea*, *Glycine max*, *Hordeum vulgare*, *O. sativa*, *Pennisetum glaucum*, *Phaseolus vulgaris*, *Saccharum officinarum*, *Solanum lycopersicum*, *Triticum aestivum*, and *Zea mays*). Orthologue and paralogue of stress-related genes product have also been incorporated in this database	http://ccbb.jnu.ac.in/stressgenes/	

Table 14.4 Software's for QTL mapping.

Tool	Description	URL	References
RiceGene Thresher	RiceGeneThresher is a powerful web-based server for mining genes underlying QTL in rice genome. It provides biologically supported evidence essential for targeting groups or networks of genes involved in controlling traits underlying QTL. It also contains information on genetic markers, genome annotation, ESTs, protein domains, gene ontology, plant stress-responsive genes, metabolic pathways and protein–protein interactions	http://rice.kps.ku.ac.th/Site/index.html	[82]
MapChart	Computer package that produces charts of genetic linkage maps and QTL data. It reads the linkage information (i.e., the locus and QTL names and their positions) from text files. MapChart program is freely available, but license is required before use. It can run on MS-Windows (95/98/ME/NT4.0/2000/XP) platform	http://www.biometris.wur.nl/UK/Software/MapChart/download/	[83]
QTL Cartographer	Suite of programs for mapping QTL on genetic linkage map. The programs use linear regression and composite and multiple interval mapping methods to dissect the underlying genetics of the quantitative traits. QTL Cartographer program is freely available and can run on MS-Windows, UNIX, and Mac platforms	ftp://statgen.ncsu.edu/pub/qtlcart/	[84]
PLABQTL	PLABQTL is a program written for the detection of loci that affect the variation in quantitative traits. Its main purpose is to localize and characterize QTL. The program employs the interval mapping approach. PLABQTL program is freely available and can run on MS-Windows, UNIX, and Mac platforms	https://www.uni-hohenheim.de/plantbreeding/software/	
QTL Network 2.0	QTLNetwork-2.0 is a user-friendly software for mapping QTL. The software is programmed by C++ programming language under Microsoft Visual C++ 6.0 environment. QTLNetwork-2.0 program is freely available and can run on Windows NT, 2000, XP, and 2003server	http://ibi.zju.edu.cn/software/qtlnetwork/	[85, 86]

Table 14.4 (*Continued*)

Tool	Description	URL	References
GridQTL (QTL Express)	QTL Express is a freely accessible web server. QTL Express is a user-friendly software for the analysis of quantitative trait data from outbred populations. This server is used for a wide variety of pedigree structures involving outbred populations, some of which are also relevant for crosses between inbred lines. The database contains tools for permutation analysis to set significance levels and bootstrap analysis to estimate confidence regions for the QTL location	http://www.gridqtl.org.uk/index.htm	[87]
MCQTL	The aim of MCQTL software package is to perform QTL mapping in a multicross design. MCQTL package is composed of three software. The first component reads the data from MAPMAKER files. The second component, ProbaPop, computes QTL genotype probabilities at specific chromosome location giving multiple marker data on each family, and stores them in XML-formatted files. The last component, Multipop, builds the pooled model and fits the observations on genotype probabilities. MCQTL program is freely available, but license is required before use, and can run on UNIX platform	http://carlit.toulouse.inra.fr/MCQTL/	[88]

14.5
Case Study: Development of QlicRice: a Web Interface for Abiotic Stress-Responsive QTL and Loci Interaction Channel in Rice

QlicRice database assists researchers to identify genes/QTL associated with abiotic stress tolerance and agricultural productivity. QlicRice provides a convenient navigation of QTL related to abiotic stress tolerance and its genomic, proteomic, and other information in a very user-friendly manner. QTL, their corresponding loci, and other details were collected from various biological databases such as Gramene (http://www.gramene.org/), TIGR (http://rice.plantbiology.msu.edu/), and other public repositories for having all information together in a single storehouse. QlicRice has a vibrant search page "Qlic Browser" with multiple search options in three query types: (i) Abiotic stress: detailed information on abiotic stresses affecting rice production; (ii) QTL Accession ID: complete details of QTL governing the abiotic stresses; and (iii) Locus ID: exploration of related loci. Autocomplete function in the search text area has been provided for easy and fast navigation by users by querying

Table 14.5 Some helpful tools and databases for plant genomics.

Type	Description	Web address	References
NCBI	National resource for molecular biology information	http://www.ncbi.nlm.nih.gov/	
Gramene QTL	Database contains a number of identified QTL from various plant crops	http://gramene.agrinome.org/qtl/	[89, 90]
TIGR	Database for rice model plant	http://rice.plantbiology.msu.edu/	[91]
TAIR	Database for *Arabidopsis* model plant	http://www.arabidopsis.org/	
PLACE	*cis*-acting regulatory DNA element finder in plants	http://www.dna.affrc.go.jp/PLACE/	[92]
Plant Transcription Factor Database	Comprehensive annotations for TF, such as functional domains, 3D structures, gene ontology, and expression information derived from ESTs and microarray	http://planttfdb.cbi.pku.edu.cn/	
Eukaryotic Promoter Database (EPD)	Annotated nonredundant collection of eukaryotic promoters, for which the transcription start site has been determined experimentally	http://www.epd.isb-sib.ch/	[93]
BLAST	Sequence alignment tool	http://blast.ncbi.nlm.nih.gov/Blast.cgi	
FASTA	Sequence alignment tool	http://www.ebi.ac.uk/Tools/fasta/	
Motif finder	A tool for identification of functional motifs	http://motif.genome.jp/	
MEME	A tool for identification of functional motifs	http://meme.sdsc.edu/meme/	[94]
Gene Evaluator (ChemGenome 1.1)	Gene identification tool	http://www.scfbio-iitd.res.in/chemgenome/index.jsp	[95]

without any accidental mistakes. It is a compendium of 974 QTL and 460 TIGR loci on different rice chromosomes related to abiotic stress. These QTL and their corresponding loci that are dispersed on different rice chromosomes have been provided in the database through 12 pie charts and physical map developed with R-statistical package (http://www.r-project.org/). Tissue-specific expression analysis can be sited to know the tissue-specific expression analysis of the QTL that are highly expressed in a particular tissue. Data on tissue-specific expression of various abiotic stress-related QTL have also been given, and it will be really useful particularly to researchers looking for promoters that are inducible but at the same time responsible for driving tissue-specific expression of the abiotic stress tolerance gene(s). The tandem repeats with their consensus sequences have also been provided in

the database, which is helpful to determine an individual's inherited traits and in the context of genomic loci/QTL evolution. Understanding the molecular functions of QTL and their role in biochemical pathways that finally determine plant phenotype during various abiotic stresses is an essential prerequisite [96]. Gene ontology analyses (http://rice.plantbiology.msu.edu/GO.retrieval.shtml) and biochemical pathway analyses by KEGG Automatic Annotation Server (http://www.genome.jp/kegg/kaas/) that assigns KEGG pathway has been used to identify the GO and KO ontologies. This information tells us about the role of the underlying genes in a metabolic pathway, in addition to the nature of their interaction with other loci. 3D structures have been included, and users can download the template structure and model, integrated in QlicRice, which have been modeled by Swiss Modeller (http://swissmodel.expasy.org/). To bridge the gap between available sequences and their unknown structure, protein structure prediction methods are important tools; thus, it is an important technique to build 3D model for further functional annotation [97–99]. As the plants experience stress at different stages of growth and development, it would be useful if the genes/QTL are identified with a specific role at a given growth stage. Several laboratories are aiming to identify such robust QTL as will remain unchanged across growth stages. Such information will be added when the database is updated on a regular basis. Moreover, owing to the global environmental changes, rise in temperature is likely to add to the existing problem of the drought affecting rice production. Hence, a deep understanding of the abiotic stress responses in crop plants on a genome-wide scale is vital for developing improved stress-tolerant crop plants [100, 101]. Overall, the QlicRice is a step forward in communicating some excellent information collected and put together in a useful database on QTL related to abiotic stresses in rice.

14.6 Conclusions and Prospects

Farmers regularly suffer from loss of crop yields due to biotic and abiotic stress factors. Development of stress-tolerant crops is a step forward in providing a tangible solution. Identification of stress-responsive genes underlying QTL is a fundamental requirement to enhanced plant tolerance to abiotic stresses. Even with rapid genome sequencing of important agricultural crops during the past decade, the execution of truthful, high-throughput phenotyping for abiotic stress tolerance traits remains a big challenge for QTL mapping studies. In recent years, a number of quantitative traits in rice (8646), sorghum (136), wheat (23), and maize (1447) have been discovered by QTL mapping and are listed at Gramene QTL database released in November 2010 (http://www.gramene.org/qtl/index.html). Scientist could enhance the progress of gene discovery for abiotic stress tolerance by effectively using the advanced genomics techniques. Through expression profiling of many genes or making whole-transcriptome chip, researchers can analyze stress-responsive gene networks, providing significant information about the role of the stress-responsive genes and their interactions with other genes. Development of

genetically improved stress-tolerant crop genotypes might be facilitated with precision by the combined use of QTL mapping and high-throughput "omics" data as discussed in this chapter.

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References

1 Levitt, J. (1980) *Responses of Plants to Environmental Stress Chilling, Freezing and High Temperature Stresses*, 2nd edn, Academic Press, New York.
2 Hasegawa, M., Hiraoka, Y., Hagiuda, J., Ogawa, M., and Aiso, S. (2002) *Gene.*, **290** (1–2), 163–172
3 Ramanjulu, S. and Bartels, D. (2002) *Plant Cell Environ.*, **25**, 141–151.
4 Zhu, J.K. (2002) *Annu. Rev. Plant Biol.*, **53**, 247–273.
5 Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M. (2003) *Curr. Opin. Plant Biol.*, **6**, 410–417.
6 Chinnusamy, V., Schumaker, K., and Zhu, J.-K. (2004) *J. Exp. Bot.*, **55**, 225–236.
7 Chinnusamy, V., Zhu, J., and Zhu, J.-K. (2007) *Trends Plant Sci.*, **12**, 444–451.
8 Salvi, S., Sponza, G., Morgante, M., Tomes, D., Niu, X., Fengler, K.A., Meeley, R., Ananiev, E.V., Svitashev, S., Bruggemann, E. *et al.* (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 11376–11381.
9 Rostoks, N., Mudie, S., Cardle, L., Russell, J., Ramsay, L., Booth, A., Svensson, J. *et al.* (2005) *Mol. Genet. Genomics*, **274**, 515–527.
10 The Arabidopsis Genome Initiative (2000) *Nature*, **408**, 796–815.
11 The International Rice Genome Sequence Project (2005) *Nature*, **436** (7052), 793–800.
12 Sasaki, T. and Burr, B. (2000) *Curr. Opin. Plant Biol.*, **3**, 138–141.
13 Yu, J., Hu, S., Wang, J., Wong, G.K., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., Zhang, X. *et al.* (2002) *Science*, **296**, 79–92.
14 Goff, S.A., Ricke, D., Lan, T.H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P. *et al.* (2002) *Science*, **296**, 92–100.
15 White, T.D., Ambrose, S.H., Suwa, G., Su, D.F., DeGusta, D., Bernor, R.L., Boisserie, J.R., Brunet, M., Delson, E., Frost, S. *et al.* (2009) *Science*, **67**, 87–93.
16 Asamizu, E., Nakamura, Y., Sato, S., and Tabata, S. (2000) *DNA Res.*, **7**, 175–180.
17 Seki, M., Narusaka, M., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., and Shinozaki, K. (2001) *Plant Physiol. Biochem.*, **39**, 211–220.
18 Seki, M., Narusaka, M., Kamiya, A., Ishida, J., Satou, M., Sakurai, T., Nakajima, M., Enju, A., Akiyama, K., Oono, Y., Muramatsu, M. *et al.* (2002) *Science*, **296**, 141–145.
19 Goff, S.A. (1999) *Curr. Opin. Plant Biol.*, **2**, 86–89
20 Ewing, R., Poirot, O., and Claverie, J.-M. (1999) *In Silico Biol.*, **1**, 18.
21 Mayer, K. and Mewes, H. (2001) *Curr. Op. Plant Biol.*, **5**, 173–177.
22 Eisen, M., Spellman, P., Brown, P., and Botstein, D. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 14863–14868.
23 Bansal, K.C., Lenka, S.K., and Tuteja, N. (2010) Abscisic acid in abiotic stress tolerance: an "omics" approach, in *Omics and Plant Abiotic Stress Tolerance* (eds N. Tuteja, S.S. Gill, and R. Tuteja), Bentham e-Books, pp. 143–150.
24 Wang, D., Lemon, W.J., and You, M. (2002) *Oncogene*, **21**, 6858–6865

25 Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y. *et al.* (2000) *Plant Cell*, **12**, 2473–3248

26 Takahashi, Y., Shomura, A., Sasaki, T., and Yano, M. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 7922–7927.

27 Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., and Yano, M. (2002) *Plant Cell Physiol.*, **43**, 1096–1105.

28 Mohan, M., Nair, S., Bhagwat, A., Krishna, T.G., Yano, M., Bhatia, C.R., and Sasaki, T. (1997) *Mol. Breed.*, **3**, 87–103.

29 McCouch, S.R. and Doerge, R.W. (1995) *Trends Genet.*, **11**, 482–487.

30 Paterson, A.H. (1996) Making genetic maps, in *Genome Mapping in Plants* (ed. A.H. Paterson), Academic Press, San Diego, pp. 23–39.

31 Bressan, R., Bohnert, H., and Zhu, J.-K. (2009) *Mol. Plants*, **2** (1), 1–2.

32 Nakashima, K. and Yamaguchi-Shinozaki, K. (2005) *JARQ*, **39** (4), 221–229.

33 Tester, M. and Bacic, A. (2005) *Plant Physiol.*, **137**, 791–793.

34 Maggio, A., Zhu, J.K., Hasegawa, P.M., and Bressan, R.A. (2006) *Plant Cell*, **18**, 1542–1557.

35 Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997) *Proc. Natl. Acad. Sci. USA*, **3**, 1035–1040

36 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) *Plant Cell.*, **8**, 1391–1406

37 Lockhart, J. and Winzeler, A. (2000) *Nature*, **405** (6788), 827–836.

38 Fizames, C.S., Munos, C., Cazettes, P. *et al.* (2004) *Plant Physiol.*, **134**, 67–80.

39 Meyers, B.C., Tej, T.H., and Vu, C.D. (2004) *Genome Res.*, **14**, 1641–1653.

40 Calsa, T. and Figueira, A. (2007) *Plant Mol. Bio.*, **63**, 745–762.

41 Chen, J., Agrawal, V., Rattray, M. *et al.* (2007) *BMC Genomics*, **8**, 414.

42 McIntosh, S., Watson, L., Bundock, P., Crawford, A., White, J., Cordeiro, G., Barbary, D., Rooke, L., and Henry, R. (2007) *Plant Biotechnol. J.*, **1**, 69–83.

43 Song, S., Qu, H., Chen, C., Hu, S., and Yu, J. (2007) *BMC Plant Biol.*, **19**, 7–49.

44 Ma, L.C., Chen, X., Jiao, L.Y. *et al.* (2005) *Genome Res.*, **15**, 1274–1283.

45 Rensink, W.S., Iobst, A., and Hart, S. (2005) *Funct. Integr. Genomics*, **5**, 201–207.

46 Oono, Y.M., Seki, M., Satou, K. *et al.* (2006) *Funct. Integr. Genomics*, **6**, 212–234.

47 Xu, W.F. and Shi, W.M. (2006) *Ann. Bot.*, **98**, 965–974.

48 Mantri, N.R., Coram, F.T., and Pang, E. (2007) *BMC Genomics*, **8**, 303.

49 Monroy, A.A., Dryanova, B., Malette, D. *et al.* (2007) *Plant Mol. Biol.*, **64**, 409–423.

50 Fernandez, P., Rienzo, J.D., Fernandez, L., and Hopp, H.E. (2008) *BMC Plant Biol.*, **8**, 11.

51 Remans, T.K., Smeets, K., Opdenakker, D. *et al.* (2008) *Planta*, **227**, 1343–1349.

52 Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. (1995) *Science*, **5235**, 484–487

53 Yamamoto, M., Wakatsuki, T., Hada, A., and Ryo, A. (2001) *J. Immunol. Methods*, **250**, 45–66.

54 Stollberg, J., Urschitz, J., Urban, Z., and Boyd, C.D. (2000) A quantitative evaluation of SAGE. *Genome Res.*, **10**, 1241–1248.

55 Mikkilineni, V., Mitra, R.D., Merritt, J., DiTonno, J.R., Church, G.M., Ogunnaike, B., and Edwards, J.S. (2004) *Biotechnol. Bioeng.*, **86**, 117–124.

56 Margulies, M. *et al.* (2005) *Nature*, **437**, 376–380.

57 Velculescu, V.E. and Kinzler, K.W. (2007) *Nat. Biotechnol.*, **25**, 878–880

58 Weber, A.P.M., Weber, K.L., Carr, K., Wilkerson, C., and Ohlrogge, J.B. (2007) *Plant Physiol.*, **144**, 32–42.

59 Torres, T.T., Metta, M., Ottenwalder, B., and Schlotterer, C. (2008) *Genome Res.*, **18**, 172–177.

60 Ren, B., Robert, F., Wyrick, J.J., Aparicio, O., Jennings, E.G., Simon, I., Zeitlinger, J., Schreiber, J., Hannett, N. *et al.* (2000) *Science*, **290**, 2306–2309.

61 Li, Z., Calcar, S.V., Qu, C., Cavenee, W.K., Zhang, M.Q., and Ren, B. (2003) *PNAS*, **100** (14), 8164–8169.

62 Cawley, S., Bekiranov, S., Ng, H.H., Kapranov, P., Sekinger, E.A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J. *et al.* (2004) *Cell.*, **4**, 499–509.

63 Pokholok, D.K., Harbison, C.T., Levine, S., Cole, M., Hannett, N.M., Lee, T.I., Bell,

G.W., Walker, K., Rolfe, P.A., and Herbolsheimer, E. (2005) *Cell*, **122**, 517–527.
64 Bernstein, B.E., Kamal, M., Lindblad-Toh, K., Bekiranov, S., Bailey, D.K., Huebert, D.J. *et al.* (2005) *Cell*, **120**, 169–181.
65 Lee, C.K., Shibata, Y., Rao, B., Strahl, B.D., and Lieb, J.D. (2004) *Nat. Genet.*, **36**, 900–905.
66 Kirmizis, A. and Farnham, P.J. (2004) *Exp. Biol. Med.*, **229**, 705–721.
67 Boguski, M.S. (2004) *Genomics*, **83**, 347–348.
68 Sanford, J.R., Wang, X., Mort, M. *et al.* (2009) *Genome Res.*, **19** (3), 381–394
69 Wong, E. and Wei, C.L. (2009) *Genome Med.*, **1**, 89.
70 Licatalosi, D.D., Mele, A., Fak, J.J. *et al.* (2008) *Nature*, **7221**, 464–469
71 Ingolia, N.T., Ghaemmaghami, S., Newman, J.R., and Weissman, J.S. (2009) *Science*, **324** (5924), 218–223
72 Chi, S.W., Zang, J.B., Mele, A., and Darnell, R.B. (2009) *Nature*, **460** (7254), 479–486.
73 Yang, J.H., Li, J.H., Shao, P., Zhou, H., Chen, Y.Q., and Qu, L.H. (2010) *Nucl. Acids Res.*, **39**, 1–8.
74 Zisoulis, D.G., Lovci, M.T., Wilbert, M.L., Hutt, K.R., Liang, T.Y., Pasquinelli, A.E., and Yeo, G.W. (2010) *Nat. Struct. Mol. Biol.*, **17**, 173–179.
75 Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995) *Science*, **270**, 467–470
76 Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. (1993) *Biotechnology*, **9**, 1026–1030.
77 Bachem, C.W., van der Hoeven, R.S., de Bruijn, S.M., Vreugdenhil, D., Zabeau, M., and Visser, R.G. (1996) *Plant J.*, **5**, 745–753
78 Yamamoto, M., Wakatsuki, T., Hada, A., and Ryo, A. (2001) *J. Immunol. Methods*, **250**, 45–66.
79 Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D.H., Johnson, D., Luo, S., McCurdy, S., Foy, M., Ewan, M., Roth, R., George, D., Eletr, S., Albrecht, G., Vermaas, E. *et al.* (2000) *Nat. Biotechnol.*, **6**, 630–634
80 Kann, M.G. (2009) *Brief. Bioinform.*, **11**, 96–110.
81 Shameer, K., Ambika, S., Varghese, S.M., Karaba, N., Udayakumar, M., and Sowdhamini, R. (2009) *Int. J. Plant Genomics*, 583429, 8.
82 Thongjuea, S., Ruanjaichon, V., Bruskiewich, R., and Vanavichit, A. (2009) *Nucleic Acids Res.*, **37** (1), D996–D1000
83 Voorrips, R.E. (2002) *J. Hered.*, **93** (1), 77–78.
84 Wang, S., Basten, C.J., and Zeng, Z.B. (2010) Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC.
85 Yang, J., Hu, C.C., Hu, H., Yu, R.D., Xia, Z., Ye, X.Z., and Zhu, J. (2008) *Bioinformatics*, **24**, 721–723.
86 Yang, J., Zhu, J., and Williams, R.W. (2007) *Bioinformatics*, **23**,1536.
87 Seaton, G., Haley, C.S., Knott, S.A., Kearsey, M., and Visscher, P.M. (2002) *Bioinformatics*, **18**, 339–340.
88 Jourjon, M.F., Jasson, S., Marcel, J., Ngom, B., and Mangin, B. (2004) *Bioinformatics*, **1**, 128–130.
89 Ware, D., Jaiswal, P., Ni, J., Pan, X., Chang, K., Clark, K., Teytelman, L., Schmidt, S., Zhao, W., Cartinhour, S., McCouch, S., and Stein, L. (2002) *Nucleic Acids Res.*, **30**, 103–105.
90 Jaiswal, P., Ni, J., Yap, I., Ware, D., Spooner, W., Youens-Clark, K., Ren, L., Liang, C., and Zhao, W. (2006) *Nucleic Acids Res.*, **34**, 717–723.
91 Ouyang, S., Zhu, W., Hamilton, J., Lin, H., Campbell, M., Childs, K., Thibaud-Nissen, F., and Malek, R.L. (2007) *Nucleic Acids Res.*, **35**, D883–D887
92 Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999) *Nucleic Acids Res.*, **1**, 297–300.
93 Schmid, C.D., Périer, V., Praz, R., and Bucher, P. (2006) *Nucleic Acids Res.*, **34**, 82–85
94 Bailey, Timothy L. and Charles, E. (1994) Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology, pp. 28–36.
95 Dutta, S., Singhal, P., Agrawal, P., Tomer, R., Kritee, Khurana, E., and Jayaram, B.J. (2006) *Chem. Inf. Mod*, **46** (1), 78–85.
96 Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. (2004) *Nucleic Acids Res.*, **32**, 277–280

97 Baker, D. and Sali, A. (2001) *Science*, **294**, 93–96.

98 Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A., and Wheeler, D.L. (2002) *Nucleic Acids Res.*, **30**, 17–20.

99 Berman, H.M., Battistuz, T., Bhat, T.N., Bluhm, W.F., Bourne, P.E., Burkhardt, K., Feng, Z., Gilliland, G.L., Iype, L., Jain, S. *et al.* (2002) *Acta Crystallogr.*, **D58**, 899–907.

100 Suh, J.P., Jeung, J.U., Lee, J.I., Choi, Y.H., Yea, J.D., Virk, P.S., Mackill, D.J., and Jena, K.K. (2010) *Theor. Appl. Genet.*, **120**, 985–995

101 Zhou, L., Zeng, Y., Zheng, W., Tang, B., Yang, S., Zhang, H., Li, J., and Li, Z. (2010) *Theor. Appl. Genet.*, **121**, 895–905.

15
Understanding Stress-Responsive Mechanisms in Plants: An Overview of Transcriptomics and Proteomics Approaches

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Plants are static in nature and, therefore, they encounter a number of biotic and abiotic stress factors during their life cycle. Plants' responses to these stress factors are differential and complex. Since the past decade, "omics" technologies are providing the major clues for understanding plant stress response mechanisms important for crop improvement. This chapter will critically evaluate the current literature on the plant transcriptomics and proteomics for understanding plant stress responses in detail in addition to the basic concept, principles, and procedure outlines of these approaches, and will also suggest important future perspectives.

15.1
Introduction

Abiotic stresses negatively impact plant growth and development and hence are the primary cause of crop loss worldwide. Plants' adaptation to these stresses is very differential and complex and depends on the activation of cascades of molecular networks involved in stress perception, signal transduction, and expression of specific stress-related genes and metabolites [1]. Although the development of "omics" technologies including transcriptomics and proteomics is in its infancy, it indeed has helped, to a great extent, unravel the possible mechanism of plant responses to a number of stress factors. The following sections will present introduction, principle and advantages, and limitations of, first, the transcriptomic and, subsequently, of the proteomic approaches in detail.

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15.2
Transcriptomic Approaches and Plant Stress Responses

In the layman's language, the transcriptome refers to the pools of RNA transcripts in a cell and the transcriptomics is the global analysis of gene expression at the RNA level and provides tools for the study of gene function. In fact, transcriptomics provides information on the presence and relative abundance of RNA transcripts and thus offers a better view of the active components in the cell than a genomic approach [2].

Transcriptomic approaches can be divided into two broad categories, hybridization-based approaches and sequencing-based approaches.

15.2.1
Hybridization-Based Approaches

15.2.1.1 Suppression Subtractive Hybridization

A detailed study involving identification and cloning of the relevant subsets of differentially expressed genes of interest is required to understand the molecular regulation of the major biological processes such as cellular growth and organogenesis. The subtractive cDNA hybridization has been a powerful approach in this regard to identify and isolate cDNAs of differentially expressed genes. This technique can be used to compare two mRNA populations and obtain cDNAs representing genes that are either overexpressed or exclusively expressed in one population compared to another. It can also be used for comparison of genomic DNA populations. In general, cDNA subtraction methods involve hybridization of cDNA from one population (tester) to excess of mRNA (cDNA) from other population (driver) and then separation of the unhybridized fraction (target) from hybridized common sequences. However, these subtraction techniques are labor-intensive, involve multiple or repeated subtraction steps, and often require more than 20 μg of poly $(A)^+$ RNA. In fact, suppression subtractive hybridization (SSH) is a PCR-based cDNA subtraction method that is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress nontarget DNA amplification.

15.2.1.1.1 **Principle** The SSH method is based on a suppression PCR effect, introduced by Lukyanov *et al.* [3]. In this method, the normalization and subtraction steps are simultaneously performed, where the normalization step equalizes the abundance of DNA fragments within the target population and the subtraction step excludes sequences that are common to the two populations being compared [4]. It is pertinent to mention here that SSH eliminates any intermediate steps demanding the physical separation of single-stranded (ss) and double-stranded (ds) DNAs, requires only one round of subtractive hybridization, and can achieve a more than 1000-fold enrichment for differentially presented DNA fragments [5] (Figure 15.1).

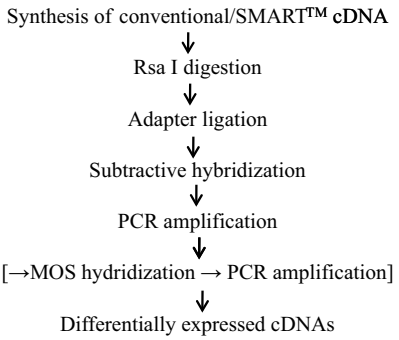

Figure 15.1 Schematic representation of the SSH procedure.

15.2.1.2 Serial Analysis of Gene Expression

*S*erial *A*nalysis of *G*ene *E*xpression (SAGE) is a method developed by Velculescu *et al*. [6] for a comprehensive analysis of gene expression patterns where it allows a rapid and detailed analysis of thousands of transcripts. SAGE technology is the second most popular high-throughput gene expression technology after microarray and does not require preexisting knowledge of the genome that is being examined and therefore SAGE can be applied to many different model systems.

Although SAGE has the capability of producing large amounts of gene expression data with the potential of providing novel insights into fundamental processes underlying (a) plant–pathogen, (b) plant–diseases, and (c) plant–stresses interactions, it has been found very effective for small-scale sequencing. Most importantly, SAGE provides an affordable and fast comparison of many experiments, stages, and so on and altogether it requires a very small amount of starting material (single-cell studies are possible).

15.2.1.2.1 **Principle** SAGE is based on three major principles: (a) a short oligonucleotide sequence, defined by a specific restriction endonuclease (anchoring enzyme, AE) at a fixed distance from the poly(A) tail, contains sufficient information to uniquely identify a mRNA transcript. As there are four nitrogen bases (A, T, G, and C), a 10 bp tag theoretically can give 4^{10} different possible sequence combinations. (b) End-to-end concatenation of short oligonucleotides form the long serial molecules that can be cloned and sequenced. (c) Quantization of the number of times a particular tag observed provides the expression level of the corresponding transcript [7] (Figure 15.2).

15.2.1.3 Microarrays

This is one of the hybridization-based approaches. A microarray is a glass microscopy slide onto which gene fragments are spotted, in the form of cDNA fragments (cDNA microarray) or *in-situ*-synthesized oligonucleotides (oligonucleotide microarray). Therefore, the microarray technology employing cDNAs or oligonucleotides is a powerful tool for analyzing gene expression profiles of plants exposed to various environmental stress factors. In fact, depending on the target nucleic acid

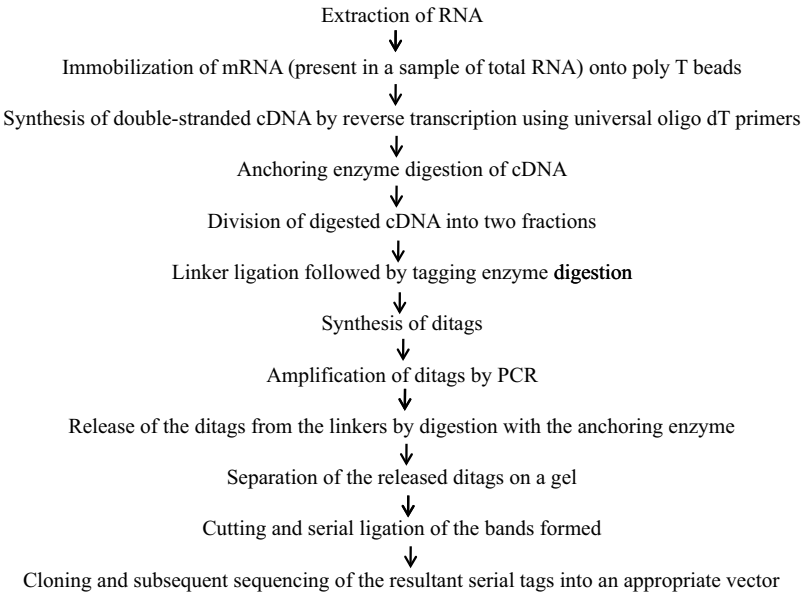

Figure 15.2 Schematic overview of major events in SAGE.

components, cDNA microarray-based technologies have been subdivided into following two formats:

i) Oligonucleotide array
ii) cDNA microarray

15.2.1.3.1 **Oligonucleotide Array** The oligonucleotide type of array consists of oligonucleotide targets, generally less than 25 mer in length, which are generated *in situ* on a solid surface by light-directed synthesis [8, 9]. Synthetic linkers modified with photochemically removable protecting groups are attached to the glass substrate. Light is then directed through a photolithographic mask to specific areas on the surface to produce localized photodeprotection. Hydroxyl-protected deoxynucleotides are incubated with the surface so that chemical coupling occurs at the sites that have been illuminated in the preceding step. By repeating these procedures with new masks, hundreds of thousands of oligonucleotides can be synthesized in a very small area [9, 10]. Alternatively, oligonucleotide arrays can be constructed by spotting presynthesized oligonucleotides on the solid surface [11–13].

Because oligonucleotide arrays are designed and synthesized on the basis of sequence information, physical intermediates such as cloning and polymerase chain reaction (PCR) are not required. Specific sequences, which are nonoverlapping if possible or minimally overlapping if necessary, can be designed to increase the hybridization sensitivity, even through their shorter sequences [10]. The oligonucleotide array is applied when more precise analysis, including the detection of single-nucleotide polymorphisms, is required [14].

15.2.1.3.2 **cDNA Microarray** The principle of microarray studies is based on the ability of an mRNA molecule to hybridize to its original DNA sequence spotted on the array. Messenger RNA is extracted from samples such as control organisms and test organisms. The mRNA is reverse transcribed to cDNA and labeled with a fluorescent label. One sample is labeled with a green fluorescent dye (Cy3), whereas the cDNA from the other sample is labeled with a red fluorescent dye (Cy5). Cy3- and Cy5-labeled samples are mixed together in equal quantities and hybridized to the microarray. The array is then scanned using laser emission. A software is used to visualize the expression levels of mRNAs of the genes and the amount of each labeled target bound to each spot on the array is quantified. Now, it has become possible to identify induced, repressed, or unchanged mRNA expression by determining the ratio of signal intensities between control and test cDNA. However, readers may consult review article by Hegde *et al.* [15] for insights into the major technical aspects of microarray fabrication, hybridization, and analysis; in addition, an article by Lettieri [16] may be useful on the applications of the microarray technique in a toxicological context.

The cDNA microarray can also be differentiated on some other ground such as the fabrication of cDNA microarray by printing cloned and amplified cDNAs onto the solid surface. Furthermore, the advantages of the cDNA microarray compared to the oligonucleotide array include less susceptibility and higher specificity due to the longer sequences of the targets [17, 18]. However, cDNA may contain repetitive sequences that are often observed in various genes, or similar sequences that are found in family member genes. These nonspecific sequences may affect the sensitivity of the cDNA microarray. The cDNA microarray can be easily used for screening steady-state mRNA expression levels [14] (Figure 15.3).

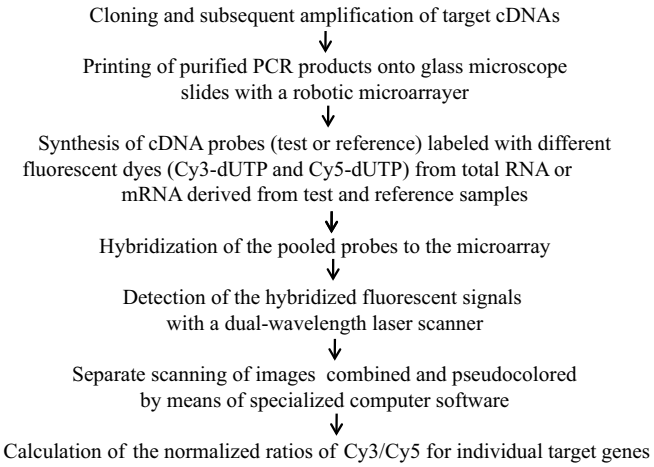

Figure 15.3 Outline of the principle of the cDNA microarray analysis system.

Advantages of Microarray Technology

- Microarray technology has largely helped in the global gene expression analysis.
- Analyses of plant defense responses.
- Microarray has been used in genomic-wide research, mutational analyses, pharmacology, toxicology, aging research, and molecular analyses of fatal diseases.

Disadvantages of Microarray Technology In addition to the significant advantages listed above, several weaknesses of microarray technology can be summarized as follows:

- High cost and time consumption, and necessity of special devices.
- Difficulty of data interchanges between individual microarrays.
- Microarray is difficult for the expression levels between individual targets to be compared in the same RNA sample (because of different hybridization rates due to variations in melting temperature depending on sequence and length of target gene fragments).

15.2.2 Sequencing-Based Approaches

Sequencing-based approaches have largely replaced the hybridization-based approaches and significantly helped gene expression analysis over the past 5 years. For the study point of view, sequencing-based approaches can be divided into two: (a) DNA sequencing of expressed sequence tag (EST) libraries and (b) next-generation sequencing (NGS).

15.2.2.1 DNA Sequencing of Expressed Sequence Tag Libraries

Expressed sequence tags represent short, unedited, and randomly selected single-pass sequence reads derived from cDNA libraries, providing a low-cost alternative (also called "poor man's genome) to whole genome sequencing, with a glimpse of the transcriptome of an organism at various stages of development. EST sequences are generated by single-pass DNA sequencing of clones randomly selected from cDNA libraries and represent partial descriptions of the transcribed portions of genomes [19]. EST sequences are widely used for a rapid and cost-effective discovery of new genes, verification of the exon–intron structure of predicted genes, and as resources for gene mapping and cDNA array construction [20]. ESTs are used as a fast and efficient method of profiling genes expressed in various tissues, cell types, or development stages [21]. One of the many interesting applications of EST database (dbEST) is gene discovery where many new genes can be found by querying the dbEST with a protein or DNA sequence.

ESTs have become an invaluable resource for gene discovery, genome annotation, alternative splicing, SNP discovery, molecular markers for population analysis, and expression analysis in animal, plant, and microbial species [22]. Although

several alternatives have been described since the emergence of EST sequencing projects, none has yet totally supplanted the use of bacterial vectors and Sanger sequencing [23].

15.2.2.2 Next-Generation Sequencing

Next-generation sequencing technologies are a set of new, state-of-the art, high-throughput sequencing technologies that came into existence after the dramatic progress in sequencing instrumentations, from Sanger-based methods, using slab gels, to capillary electrophoresis (CE). In fact, the NGS technologies differ from conventional capillary-based sequencing in that NGS has departed from Sanger sequencing chemistry and sequencing is often performed on templates formed as beads or spots of DNA [24]. NGS platforms are being utilized for targeted sequencing of candidate genes or genomic intervals to perform sequence-based association studies.

Several NGS technologies have recently emerged that can be discussed under following subheadings:

- Pyrosequencing
- Fluorescent-labeled sequencing by synthesis
- Sequencing by hybridization and ligation, and microchip-based CE

15.2.2.2.1 **Pyrosequencing** The pyrosequencing technique is based on the process of sequencing by synthesis. It was developed by 454 Life Sciences and Roche Applied Science.

Principle Pyrosequencing is based on the principle that when a nucleotide is incorporated into the growing DNA strand, the pyrophosphate is released, which is subsequently converted to ATP by enzyme; a light is produced when ATP comes in contact with enzyme luciferase (Luc). Individual and sequential addition of dNTPs to the growing DNA molecules takes place. The incorporation of a nucleotide emits the flash of light signals that can be easily correlated with the incorporation of specific nucleotide.

454 Life Sciences has developed several machines for pyrosequencing. Genome Sequencer (GS)-20 was the first next-generation DNA sequencer on the market released in 2005. Margulies *et al.* [25] reported that GS-20 was able to read up to 25 million bases of bacterial genome in a single 4 h run. The Genome Sequencer FLX (GS-FLX) was released in 2007. This instrument is able to read lengths of 250 bases and is able to perform mate-paired reads. In addition, an average of 100 million DNA bases can be sequenced in a 7.5 h run [26]. In 2008, 454 Life Sciences launched the GS-FLX Titanium series reagents for use on the present instrument, with the ability to sequence 400–600 million base pairs with 400–500 base pair read lengths. With its high accuracy, low cost, and long reads, many researchers have switched from traditional Sanger capillary sequencing instruments over to the 454 sequencing platform for a variety of genome projects.

15.2.2.2.2 **Fluorescent-Labeled Sequencing by Synthesis** Genome Analyzer (GA) system developed by Illumina uses a polymerase-based sequencing-by-synthesis

(SBS) chemistry. This platform utilizes fluorescent-labeled and reversible terminator chemistry, unlike the instrument by 454 Life Sciences, but produces read lengths of approximately 50 bp and >2000 Mb of sequence data per run over the course of approximately 4 days [24]. In addition, Illumina's GA system can be used in gene expression, SNP discovery, base resequencing, and ChIP experiments (ChIP-seq).

15.2.2.2.3 **Sequencing by Hybridization and Ligation, and Microchip-Based Capillary Electrophoresis** The instrument based on the method of sequencing by hybridization followed by ligation was developed by Applied Biosystems (ABI) generally called sequencing by oligonucleotide ligation and detection (SOLiD) [27]. In fact, The SOLiD technology platform uses emulsion PCR and sequencing by oligonucleotide ligation and detection. SOLiD technology can be potentially used in gene expression analysis and other approaches. The overall accuracy rate for the SOLiD system is greater than 99.94% and this applies to paired end runs that produce 50 bp reads.

The microchip-based capillary electrophoresis-based sequencing systems involve the separation of fluorescent-labeled sequencing samples on hair-thin, 30–50 cm long capillary gels. CE array chips have been fabricated on the basis of the well-understood behavior of a single-channel chip system. Different materials, for example, silicon [28], glass [29], and plastics [30, 31] have been used. A variety of different fabrication processes have also been developed to accommodate the complicated requirements and materials used for making such a device. Microchip-based CE systems have demonstrated use in diverse applications such as the separation of amino acids [32], analysis of blood serum cortisol [33], examination of polymerase chain reaction amplicons [34], and analysis of metal–ion complexes [35]. However, readers are advised to consult recent, excellent reviews by Hert *et al*. [26] and Simon *et al*. [24] for a detailed working principle and application of ABI-SOLiD and CE array-based sequencing.

Almost all the instruments for next-generation sequencing are able to generate three to four orders of magnitude more sequences and are considerably less expensive than the Sanger method on the ABI 3730xL platform (hereafter referred to as ABI Sanger) [36–39]. These next-generation sequencing methods promise a cost-effective means of either deeply sampling or fully sequencing an organism's transcriptome, with even small experiments tagging a very large number of expressed genes.

15.3
Proteomic Approaches in Plant Stress Responses

The study of complex biological questions through comparative proteomics is becoming increasingly attractive to plant biologists. Since the past few decades, the major aim of proteomic studies is to decipher the constituents of a proteome, thus to reveal the basic mechanism of plant responses to various environmental stresses by analyzing changes and the dynamics of changes on the protein level. It is pertinent to mention here that attention has been focused on the determination of the function

and functional network of proteins by proteome analysis after the completion of genome sequences of several organisms. Although sequence analysis of genomic DNA started in the 1990s on a full scale, developed rapidly during the past decade, and made available the entire sequence of genomic DNA for many organisms including higher plants, animals, and human, and the expression of genes can be analyzed at the transcriptional level, the expression of proteins cannot always be analyzed from gene expression because there exists a relatively low correlation (correlation coefficient about 0.5) in quantity between mRNA and protein [40, 41]. Moreover, information on protein posttranslational modification, structure, and protein–protein interaction cannot be provided by the DNA sequence and/or the expression of mRNA. In addition, almost all proteins are posttranslationally modified and then form specific structure and function through protein–protein (ligand) interaction. Therefore, the analysis of proteins assumes great importance. Although proteome research started after the genome sequence analysis was accomplished, the developments over the last few years have been remarkable [41]. The systematic analysis of proteins in plants has greatly helped researchers to understand gene functions through complementation of gene and gene expression analysis in detail. Recently, the development of advanced techniques for revealing coding genes of the organism under study, gene annotation, and functional characterization have added great momentum to plant proteome analysis.

Proteomic analysis is a multistep process that typically involves protein extraction, fractionation, separation, and mass spectrometry (MS). However, a classical proteomics work involves the following two major steps: (i) *separation of proteins step*, usually 2-dimensional gel electrophoresis (2DE), and (ii) *identification of separated proteins step*, usually mass spectrometry.

The following sections will review various gel- and nongel-based approaches that are used in a wide range of biological systems for studying differentially expressed proteins including multidimensional protein identification and labeled or nonlabeled approaches.

15.3.1
Gel-Based Approaches

Gel-based proteomics generates qualitative and quantitative protein behavioral data and as such it provides a core technology to integrate information produced using various "omic" technologies.

15.3.1.1 One- or Two-Dimensional Polyacrylamide Gel Electrophoresis

Depending on the plane of separation, the gel-based approaches for proteome analysis may be one or two dimensional. The 1-dimensional electrophoresis (1DE) is used for most routine protein and nucleic acid separations. The support medium for electrophoresis can be formed into a gel within a tube or it can be layered into flat sheets. In general, the tubes are used for easy 1DE separations.

O'Farrell in 1975 [42] first described the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). This is the simplest, most popular, and versatile method

of protein separation among a rapidly growing array of comparative proteomic technologies. In fact, 2DE allows for the separation of complex protein and is based on the orthogonal separation of proteins according to their isoelectric points through isoelectric focusing and molecular mass separation using SDS-polyacrylamide gel electrophoresis (PAGE). An SDS-PAGE is run first in one direction and then again at right angles. In the first dimension, an isoelectric focusing (IEF) gel is run and in the second dimension the proteins are separated in SDS-PAGE. A greater number of individually different proteins can be resolved in a highly repeatable fingerprint-like pattern. Two-dimensional gel-based strategies separate intact proteins on the basis of both charge (isoelectric point, p*I*) and mass, and therefore have the ability to resolve multiple charged isoforms (that may result from phosphorylation or other charged posttranslational modifications) and biologically significant proteolytic products.

Advantages of 2DE

i) Represents entire proteome.
ii) Can resolve up to 5000 different proteins simultaneously (≈2000 proteins routinely).
iii) It can detect and quantify <1 ng of protein per spot.
iv) Provides more than a raw list of proteins, and also intensities.
v) Can track posttranslational modifications.
vi) Preset conditions can be manipulated to enhance resolution (pH ranges, size of gel, staining methods, solubility, etc.).
vii) Delivers a map of intact proteins that can be stored and analyzed at will.

Limitations of 2DE

i) Reproducibility and sensitivity are less.
ii) Poor resolution of hydrophobic or membrane-bound and nuclear proteins.
iii) Sample loading/sample size capacity can limit experiments.
iv) Hard to resolve very acidic and/or very basic proteins (pH range from 2.5 to 12), very small, or very large proteins.
v) Difficult to automate process or create accurate databank standards.
vi) Only highly abundant proteins from total cell lysates are visualized and low-abundance proteins of physiological relevance, such as regulators or signaling proteins, are difficult to detect.

The introduction of immobilized pH gradients (IPGs) has largely overcome the major shortcomings listed above for the first dimension of 2DE [43]. A pH gradient formed by mixtures of acrylamide buffers is covalently fixed to the acrylamide matrix during gel polymerization. The gradient does not drift and cannot be distorted. Here, a series of chemically well-defined acrylamide derivatives with the general structure $CH_2{=}CH{-}CO{-}NH{-}R$ (where, R contains either a carboxyl or an amino group) are used that form a series of buffers with different p*K* values ranging between 1 and 13. A true steady-state IEF with increased reproducibility is allowed because of the

generation of extremely stable pH gradients due to occurrence of copolymerization of the reactive end with the acrylamide matrix. With this improved first dimension, not only a substantially wider spectrum of proteins can be resolved throughout the entire pH gradient in one gel but also lower abundance proteins caused by increased sample loading capacity can be detected.

Significant improvements to the 2DE are being made with the advancement of new technologies such as (a) the use of IPG DryStrip technology, (b) the use of semiautomated devices such as the IPGphor in the first dimension, and (c) the use of multiple SDS-PAGE apparatus for running up to 20 different samples in parallel in the second dimension. With the application of fluorescent dyes or isobaric tags, now we can have (a) improved solubilization and (b) separation of hydrophobic proteins, (c) display of low abundance proteins, and (d) reliable protein quantization. In addition, the use of multiplexed fluorescent Cy-Dye staining of different proteome states in difference gel electrophoresis (DIGE) technology has largely eliminated the technical irreproducibility of 2DE.

Unlu *et al.* [44] first introduced the two-dimensional differential gel electrophoresis (2D-DIGE) technology. 2D-DIGE uses three spectrally resolvable fluorescent dyes (Cy2, Cy3, and Cy5) to label up to three samples to be run together on the same 2D gel, adds an essential quantitative component to 2D-GE, and allows for the detection of subtle changes in protein abundance with statistical confidence. As discussed above, a classical 2DE approach lacks the intrinsic gel-to-gel variation that requires several replicate gels of each sample that are not directly overlapped. With the use of multiplexing methods such as fluorescent 2D-DIGE, substantial variability can be reduced by displaying two or more complex protein mixtures labeled with different fluorescent dyes in a single 2D gel. In addition, the use of spectrally resolvable fluorescent dyes also renders 2D-DIGE much more quantitative than colorimetric methods. The detection of proteins in samples in 2D-DIGE has a large dynamic range of 10^4–10^5, and here the dye sensitivity is capable of detecting 0.25–1 ng of sample, thus enabling the detection of relatively low copy-number proteins. Therefore, with this excellent sensitivity, DIGE can be used to analyze relatively small amounts of even very complex cell extracts.

It is pertinent to mention here the other gel-based approach in which metabolic labeling of proteins is done using radioactive isotope-labeled amino acids, and 2DE and recording are done on color negative film by radiographic exposure [45]. Spandidos and Rabbitts [46] described another gel-based subproteome differential display method in which the radiolabelled proteins are used from one source and silver-stained proteins from a second source, which are mixed in a gel in a 1 : 100 ratio, to allow the precise discrimination between members of each subproteome (chromatographic fractions) using commonly available software. In the same year, Gerner *et al.* [47] developed a quantitative proteome profiling method where precise quantitation both of the protein amount and of the ^{35}S incorporated is allowed using a combination of radiolabeling and SYPRO ruby staining of the same gels. In addition, this method also determines the absolute values of cell protein amounts, as well as synthesis and turnover rates.

15.3.2 Nongel-Based Approaches

It is evident from above discussion on gel-based approaches for proteome analysis that most of the classical proteomics approaches suffer from a number of limitations. The identification of proteins by mass spectrometry was made possible by the development of "soft" ionization techniques, namely, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) developed in the late 1980s in Europe by Michael Karas and Franz Hillenkamp and in the United States by John Fenn, respectively. MS is now firmly entrenched as the method of choice for both protein identification and characterization of posttranslational modifications. In addition, MS has become an increasingly attractive analytical instrument for biologists due in part to new ionization methods and major improvements in mass accuracy, resolution, sensitivity, and ease of use. Therefore, with the introduction of various nongel-based approaches for proteomics studies, we can achieve a dynamic range of analysis (usually 10^3–10^5) and identify low-concentration proteins. In fact, the most of nongel-based approaches digest complex mixtures of proteins in solution where the resulting peptide mixture is fractionated by one or several steps of capillary chromatography and analyzed in a data-dependent manner by MS/MS. We may summarize the major steps in MS as follows: MS consists of (i) an ion source, (ii) the mass analyzer, and (iii) an ion detection system. Analysis of proteins by MS occurs in three major steps: (a) protein ionization and generation of gas-phase ions, (b) separation of ions according to their mass to charge ratio, and (c) detection of ions. In nongel-based approaches such as isotope-coded affinity tag (ICAT) and multidimensional protein identification technology (MudPIT), samples are directly analyzed by MS, whereas in gel-based proteomics (2DE and 2D-DIGE), the protein spots are first excised from the gel and then digested with trypsin. The resulting peptides are then separated by liquid chromatography (LC) or directly analyzed by MS. The experimentally derived peptide masses are correlated with the peptide fingerprints of known proteins in the databases using search engines (e.g., Mascot and Sequest).

15.3.2.1 One-Dimensional LC-MS/MS Technology

Identification of proteins is required for understanding the complex and highly dynamic proteome of a cell/tissue/organ/organism. For this purpose, mass spectroscopy has been widely used. However, mass spectrometers alone cannot resolve more than a certain number of ion signals, therefore, before identification of proteins, reduction of sample complexity by using an advanced separation technique is necessary. Mass spectroscopy coupled with liquid chromatography now has become a method of choice for identification of proteins present in a complex proteomic sample. LC-MS/MS is an advanced technique that combines the separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry.

15.3.2.1.1 Principle In LC-MS/MS, complex protein mixtures are first digested into small peptides that are separated by liquid chromatography. It is important to note that this method is based on peptide separation, instead of protein separation,

because peptides in complex mixtures exhibit a more uniform behavior than individual subclasses of proteins [48]. It is also important that these small peptides can be easily ionized in the mass spectrometer in comparison to the large proteins.

For single-dimensional separation of protein digests, i.e., peptides by liquid chromatography, a nanocolumn packed with reverse-phase C18 resin is used. Peptides bind to C18 chain by hydrophobic interactions. After loading onto the reverse-phase C18 resin nanocolumn, they are directly eluted into the ionization chamber of mass spectrometer.

For ionization, generally electrospray method is used. These ionized peptides are first detected as charged ions that are separated by mass/charge ratio. Peptide with a specific mass/charge is then selected and further fragmented using "collision-induced dissociation" [49, 50]. It takes place in a collision cell filled with N_2 gas with a certain pressure. The selected peptide ion is excited by applying a certain voltage and then it undergoes a collision-induced dissociation by energetic collision with N_2 molecules. After that, they are sent through a second mass spectrometer that scans and detects fragmentation pattern. This CID fragmentation pattern is used to determine the sequence of the peptide and this sequence information is then used to search against databases using computer software for protein identification. This approach is known as MS/MS technique or tandem mass spectroscopy (Figure 15.4).

Advantages of One-Dimensional LC-MS/MS Technology LC-MS/MS is a superior method for protein identification. Identification of proteins in a complex mixture of more than 50 analytes can be achieved without prior purification by this technology (51–53). Its advantages are as follows:

i) Its sensitivity is high because of the use of nanocolumn which can concentrate peptides many folds before detection by MS/MS.
ii) All amount of the sample loaded on a LC-MS/MS system is utilized during ionization process, therefore for detection of a less abundant protein, more amount of sample can be utilized.
iii) Another advantage of LC-MS/MS is its reliability. Confidence level of protein identification is high as it is based on MS/MS sequencing of only one peptide.

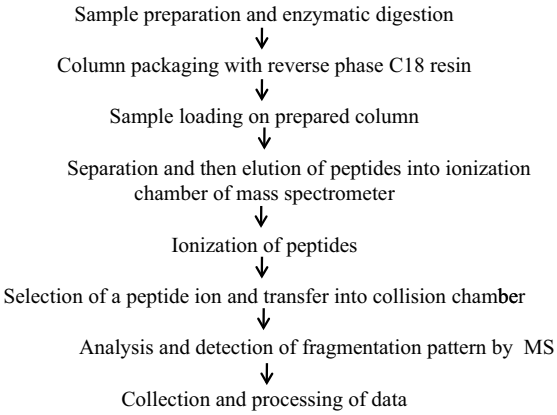

Figure 15.4 Schematic representation of the one-dimensional LC-MS/MS technology.

Limitations of One-Dimensional LC-MS/MS Technology The limitations of this technology are as follows:

i) This technique is routinely used to identify proteins directly from complex mixtures but its sensitivity is limited by the separation capacity of 1D-LC technique.
ii) In LC-MS/MS, data acquisition, database search, and search result analysis may take several hours which is time-consuming.

15.3.2.2 Multidimensional Protein Identification Technology

MudPIT was developed by Washburn *et al.* in 2001 [54]. It is an advanced technology for large-scale analysis of proteome. In general, MudPIT is a combination of two or multidimensional liquid chromatography systems with tandem mass spectrometer.

15.3.2.2.1 **Principle** In this technology, a mixture of proteins is first subjected to reduction, alkylation, and digestion. Reduction breaks cysteine disulfide bonds, alkylation prevents reformation of these bonds, and digestion converts the protein mixture to a mixture of peptides. The digested sample is directly loaded onto a nanocolumn with an internal diameter of 50–100 μm that has a tip with an inner diameter of 2–5 μm [55–58]. The nanocolumn is packed with C18 resin followed by strong cation exchange (SCX) resin. This is known as biphasic column. Desalting of samples containing high salt concentration is required before loading onto this biphasic column. Alternatively, a triphasic column containing C18 resin, SCX resin, and C18 resin in a sequential manner is also generally used. After loading, the column is attached to the tandem mass spectrometer. A high-performance liquid chromatography (HPLC) pump is used to supply different buffers through the column for separation and elution of peptides. In a triphasic column, peptides are desalted in the first step by C18 resin and then they are eluted onto the SCX phase [59]. In SCX, separation is based upon charge. Peptides of similar isoelectric point are sequentially advanced to next C18 resin where separation takes place on the basis of size and hydrophobicity. Thus, peptides are stepwise separated using SCX and C18 resins. After separation and elution from the nanocolumn, peptides are ionized by ESI method and then subjected to the mass spectrometer, where they are separated on the basis of their mass-to-charge ratio (m/z). Selected peptide ions are fragmented via collision-induced dissociation in the tandem mass spectrometer. Tandem mass spectra are generated and are searched against a protein database to determine the peptide sequence and their proteins [53, 60] (Figure 15.5).

Advantages of Multidimensional Protein Identification Technology The use of MudPIT has rapidly increased in proteomics research as it has revolutionized large-scale analysis of complex proteome. It is unbiased, as proteins of extreme values of molecular weights, pI, hydrophobicity, and abundance can be identified with equal sensitivity. MudPIT has been used in a wide range of proteomics experiments [61–66]. These include the following:

i) Identification of protein complexes.
ii) Profiling of organelle/membrane/cell/tissue-specific proteins.

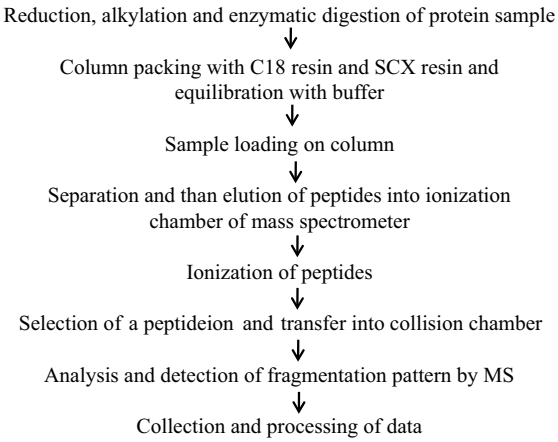

Figure 15.5 Schematic representation of the multidimensional protein identification technology.

iii) Identification of posttranslational modifications.
iv) Quantitative comparison of protein expression level by coupling this technology to labeling methods such as stable isotope labeling, SILAC or iTRAQ [67, 68].

Limitations of Multidimensional Protein Identification Technology The success of MudPIT greatly depends upon the chromatographic separation of mixtures of peptides. The limitations of MudPIT are as follows:

i) Requirement of high-quality nanocolumn and solvents of highest purity.
ii) MudPIT column is a nanocolumn; therefore, it has a limited sample loading capacity.
iii) Successful packing of column for good reproducibility is required.
iv) MudPIT analysis of complex proteome generates huge amount of data. For analysis of such data, advanced computational tools are necessary, and this analysis step can take time from few hours to few days depending upon the sample complexity, the size of database being searched, and the computational tools being used.

15.3.3
Labeled or Nonlabeled Approaches

Mass spectrometry-based methods have become popular not only for qualitative but also for quantitative analysis of a proteome. Information on what types of proteins are expressed in a proteome and what is the level of expression of these proteins is also important.

In classical quantitative proteomics, proteins are separated by one-/two-dimensional polyacrylamide gel electrophoresis and then identified by mass spectrometry [69]. Here, quantification is based upon intensity of staining of a protein. Another technique 2D-DIGE provides more precise quantification as samples to be compared are run together on the same gel; therefore, errors due to separate gel runs get removed [70]. These classical approaches have gel-based limitations such as low-

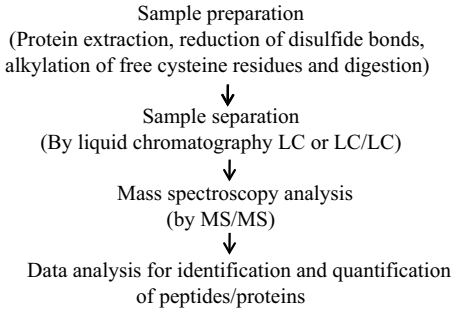

Figure 15.6 Outline of the procedure of the MS-based quantitative methods.

resolution protein separation and difficulty in identification of proteins of extreme molecular weights and p*I* values and that of low solubility [71–73]. These limitations have been overcome by modern mass spectrometry-based methods. Two types of MS-based quantification methods have been developed for extensive comparison of multiple proteomes (Figure 15.6):

1) Labeled quantitative methods
2) Label-free quantitative methods

In labeled quantitative methods, samples to be compared are first separately labeled with different isotopes after which they are pooled together and then they are subjected to sample preparation, separation, and analysis by MS/MS. While in case of label-free quantitative methods, each sample is separately prepared, separated, and analyzed.

15.3.3.1 Principle of Quantification

In label-free quantitative methods, quantification is based on "spectral counting" [74]. Spectral counting is based upon the number of times a particular peptide is identified by MS/MS, which is directly proportional to abundance of corresponding protein present in the sample.

In labeled quantitative methods, during separation by liquid chromatography differentially labeled peptides elute simultaneously, but due to mass difference, two forms of a peptide can be detected by mass spectroscopy. Quantification is done by comparing their signal intensities [75–77]. Intensity ratio of deferentially labeled peptides is calculated and subsequently the fold change in the ratio of peptide and protein is calculated. Thus, relative quantification is performed by labeling approaches.

15.3.3.2 Types of Methods

Labeling approaches include15N/14N metabolic labeling [78], stable isotope labeling by amino acids in cell culture (SILAC) [79], 18O/16O enzymatic labeling, ICAT [75], isotope-coded protein labeling (ICPL) [80], isobaric tags for relative and absolute quantification (iTRAQ) [81], tandem mass tags (TMTs) [82], and other chemical labeling. In case of label-free approaches, normal LC-MS and LC-MS/MS are widely used methods [83, 84].

Although labeling methods have provided high delectability and reproducibility of protein quantification, they have some limitations including complexity in sample

preparation, requirement of high amount of sample, expensive reagents, increased time span and specific computational tools, and importantly incomplete labeling of samples. Therefore, label-free methods being cleaner and relatively cheaper are gaining more interest [78, 85].

15.3.4 Data Mining Tools

Software is used for analysis of a large amount of data generated by modern mass spectroscopy-based techniques. Different search methods are used for peptide identification. These methods can be divided into three types: peptide mass fingerprint, sequence query, and MS/MS ion search. Digestion of a protein using a specific enzyme results in mixture of peptides whose mass spectrum provides a fingerprint of such specificity that protein identification is possible. Therefore, it is known as peptide mass fingerprinting. But it has one drawback that only proteins with already known sequence can be identified. In sequence query method, molecular mass information of peptides is combined with sequence, composition, and fragment ion data. The source of information about sequence is the analysis of a series of peaks of an MS/MS spectrum. This method was developed by Mann and Wilm in 1994 [86]. In MS/MS, ions search data are accepted in different types of peak list formats. A single MS/MS spectrum or a multidimensional LC-MS/MS run containing data from many thousands of peptides may be searched.

Peptide identification algorithms fall into two broad classes: database search and *de novo* search. The former search takes place against a database containing all amino acid sequences assumed to be present in the analyzed sample, whereas the latter infers peptide sequences without knowledge of genomic data. SEQUEST, Mascot, X! Tandem, Phenyx, OMSSA, MyriMatch, Graylag, ByOnic, InsPecT, SIMS, and MassWiz are some database search algorithms used for identification of peptides. For *de novo* sequencing, DeNoS, PEAKS, and Lutefisk algorithms are used.

15.4 Conclusions and Prospects

Advanced new technologies for proteomic analysis have accelerated biological research. Improvement became possible because of the development of advanced separation processes, mass spectrometers, and computer software tools. This has helped us in making new discoveries in the field of proteomics. However, further improvement in proteomics techniques is required in some fields such as quantitative analysis of posttranslational modifications. It will not be wrong to say that there is a huge scope for further improvement of proteomic technology so that the comprehensive analysis of complex biological processes can be done. Because new techniques generate large volume of data in exponential manner, there is need to develop new statistical tools required for finding out logical interpretations of the data. Finally, as these techniques will be developed and popularized, more and more biological information will be dug out.

Acknowledgments

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References

1 Vinocur, B. and Altman, A. (2005) *Curr. Opin. Biotechnol.*, **16**, 123–132.
2 Jamers, A., Blust, R., and Coen, W.D. (2009) *Aqu. Toxicol.*, **92**, 114–121.
3 Lukyanov, S.A., Gurskaya, N.G., Lukyanov, .K.A. *et al.* (1994) *Russ. J. Bioorg. Chem.*, **20**, 701–704.
4 Gurskaya, N.G., Diatchenko, L., Chenchik, A. *et al.* (1996) *Anal. Biochem.*, **240**, 90–97.
5 Rebrikov, D.V. (2008) *Cold Spring Harb. Protoc.* doi: 10.1101/pdb.top21
6 Velculescu, V.E., Zhang, L., Vogelstein, B. *et al.* (1995) *Science*, **270**, 484–487.
7 Tuteja, R. and Tuteja, N. (2004) *Bioessays*, **26**, 916–922.
8 Fodor, S.P.A., Read, J.L., Pirrung, M.C. *et al.* (1991) *Science*, **251**, 767–773.
9 Hacia, J.G., Edgemon, K., Fang, N. *et al.* (1996) *Hum. Mutat.*, **16**, 354–363.
10 Lipshutz, R.J., Fodor, S.P., Gingeras, T.R. *et al.* (1999) *Nat. Genet.*, **21**, 20–24.
11 Yershov, G., Barsky, V., Belgovskiy, A. *et al.* (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4913–4918.
12 Marshall, A. and Hodgson, J. (1998) *Nat. Biotechnol.*, **16**, 27–31.
13 Ramsay, G. (1998) *Nat. Biotechnol.*, **16**, 40–44.
14 Lindblad-Toh, K., Winchester, E., Daly, M.J. *et al.* (2000) *Nat. Genet.*, **24**, 381–386.
15 Hegde, P., Qi, R., Abernathy, K., Gay, C. *et al.* (2000) *Biotechniques*, **29**, 548–556.
16 Lettieri, T. (2006) *Env. Health Perspec.*, **114**, 4–9.
17 Bilban, M., Head, S., Desoye, G. *et al.* (2000) *Placenta*, **21** (Suppl A), S99–S105
18 Iida, K. and Nishimura, I. (2002) *Crit. Rev. Oral Biol. Med.*, **13**, 35–50.
19 Nagaraj, S.H., Gasser, R.B., and Ranganathan, S. (2007) *Brief Bioinform.*, **8**, 6–21.
20 Lee, B., Hong, T., Byun, S.J. *et al.* (2007) *Nucleic Acids Res.*, **35**, W159–W162.
21 Adams, M.D., Kelly, J.M., Gocayne, J.D. *et al.* (1991) *Science*, **252**, 1651–1656.
22 Bouck, A. and Vision, T. (2007) *Mol. Ecol.*, **16**, 907–924.
23 Wall, P.K., Leebens-Mack, J., Chanderbali, A.S. *et al.* (2009) *BMC Genomics*, **10**, 347.
24 Simon, S.A., Zhai, J., Nandety, R.S. *et al.* (2009) *Annu. Rev. Plant Biol.*, **60**, 305–333.
25 Margulies, M., Egholm, M., Altman, W.E. *et al.* (2005) *Nature*, **437**, 376–380.
26 Hert, D.G., Fredlake, C.P., and Barron, A.E. (2008) *Electrophoresis*, **29**, 4618–4626.
27 Shendure, J., Porreca, G.J., Reppas, N.B. *et al.* (2005) *Science*, **309**, 1728–1732.
28 Webster, J.R. and Mastrangelo, C.H. (1997) *Transducers*, **97**, 503–506.
29 Wooley, A.T. and Mathies, R.A. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 348–352.
30 McCormick, R.M., Nelson, R.J., Alonso-Amigo, M.G. *et al.* (1997) *Anal. Chem.*, **69**, 2626–2630.
31 Xie, W., Yang, R., Xu, J. *et al.* (2000) *Methods Mol. Biol.*, **162**, 67–83.
32 Jacobson, S.C., Hergenroder, R., Moore, A.W. *et al.* (1994) *Anal. Chem.*, **66**, 4127–4132.
33 Koutny, L.B., Schmalzing, D., Taylor, T.A. *et al.* (1996) *Anal. Chem.*, **68**, 18–22.
34 Cheng, J., Waters, L.C., Fortina, P., Hvichia, G.E. *et al.* (1998) *Anal. Biochem.*, **257**, 101–105.
35 Fan, Z.H. and Harrison, D.J. (1994) *Anal. Chem.*, **66**, 177–184.
36 Bonetta, L. (2006) *Nat. Methods*, **3**, 141–147.

37 von Bubnoff, A. (2008) *Cell*, **132**, 721–723.
38 Schuster, S.C. (2008) *Nat. Methods*, **5**, 16–18.
39 Harismendy, O., Ng, P.C., Strausberg, R.L. *et al.* (2009) *Genome Biol.*, **10**, R32.
40 Anderson, L. and Seilhamer, J. (1997) *Electrophoresis*, **18**, 533–537.
41 Hirano, H., Islam, N., and Kawasaki, H. (2004) *Phytochem.*, **65**, 1487–1498.
42 O'Farrell, P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.
43 Görg, A., Obermaier, C., Boguth, G. *et al.* (2000) *Electrophoresis*, **21**, 1037–1053.
44 Unlu, M., Morgan, M.E., and Minden, J.S. (1997) *Electrophoresis*, **18**, 2071–2077.
45 Goldman, R.C., Trus, B.L., and Leive, L. (1983) *Eur. J. Biochem.*, **131**, 473–480.
46 Spandidos, A.O. and Rabbitts, T.H. (2002) *J. Mol. Biol.*, **318**, 21–31.
47 Gerner, C., Vejda, S., Gelbmann, D. *et al.* (2002) *Mol. Cell. Proteom.*, **1**, 528–537.
48 Lohrig, K. and Wolters, D. (2009) *Methods Mol. Biol.*, **564**, 143–153.
49 Hayes, R.N. and Gross, M.L. (1990) *Methods Enzymol.*, **193**, 3–36.
50 McLuckey, S.A. (1992) *J. Am. Soc. Mass Spec.*, **3**, 599–614.
51 McCormack, A.L., Eng, J.K., and Yates, J.R., III (1994) *Methods*, **6**, 274–283.
52 McCormack, A.L., Schieltz, D.M., Goode, B. *et al.* (1997) *Anal. Chem.*, **69**, 767–776.
53 Eng, J., McCormack, A., and Yates, J.R., III (1994) *J. Am. Mass Spectrom.*, **5**, 976–989.
54 Washburn, M.P., Wolters, D., and Yates, J.R., III (2001) *Nat. Biotechnol.*, **19**, 242–247.
55 Griyn, P.R., CoVman, J.A., Hood, L.E. *et al.* (1991) *Int. J. Mass Spectrom. Ion Process.*, **111**, 131–149.
56 Gatlin, C.L., Kleeman, G.R., Hays, L.G. *et al.* (1998) *Anal. Biochem.*, **263**, 93–101.
57 Emmet, M.R. and Caprioli, R.M.J. (1994) *J. Am. Soc. Mass Spectrom.*, **5**, 605–613.
58 Davis, M., Stahl, D., Hefta, S. *et al.* (1999) *Anal. Chem.*, **67**, 4549–4556.
59 McDonald, W.H., Oni, R., Miyamoto, D.T. *et al.* (2002) *Int. J. Mass Spectrom.*, **219**, 245–251.
60 Sadygov, R.G., Cociorva, D., and Yates, J.R., III (2004) *Nat. Methods*, **1**, 195–202.
61 Cagney, G., Park, S., Chung, C., Tong, B. *et al.* (2005) *J. Proteome Res.*, **4**, 1757–1767.
62 Yates, J.R., III, Gilchrist, A., Howell, K.E. *et al.* (2005) *Nat. Rev.*, **6**, 702–714.
63 Klose, J. and Kobalz, U. (1995) *Electrophoresis*, **16**, 1034–1059.
64 Cantin, G.T., Venable, J.D., Cociorva, D. *et al.* (2006) *J. Proteome Res.*, **5**, 127–134.
65 Link, A. (2002) *Trends Biotechnol.*, **20**, S8–S13.
66 Fujii, K., Nakano, T., Kawamura, T., Usui, F. *et al.* (2004) *J. Proteome Res.*, **3**, 712–718.
67 Maurya, P., Meleady, P., Dowling, P. *et al.* (2007) *Anticancer Res.*, **27**, 1247–1255.
68 Washburn, M.P., Koller, A., Oshiro, G. *et al.* (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 3107–3112.
69 Issaq, H.J. and Veenstra, T.D. (2008) *BioTechniques*, **44**, 697–700.
70 Kondo, T. (2008) *J. Biochem. Mol. Biol.*, **41**, 626–634.
71 Rabilloud, T., Adessi, C., Giraudel, A. *et al.* (1997) *Electrophoresis*, **18**, 307–316.
72 Righetti, P.G., Bossi, A., Gorg, A., Obermaier, C. *et al.* (1996) *J. Biochem. Biophys. Methods*, **31**, 81–91.
73 Harder, A., Wildgruber, R., Nawrocki, A. *et al.* (1999) *Electrophoresis*, **20**, 826–829.
74 Liu, H., Sadygov, R.G., and Yates, J.R., III (2004) *Anal. Chem.*, **76**, 4193–4201.
75 Gygi, S.P., Rist, B., Gerber, S.A. *et al.* (1999) *Nat. Biotechnol.*, **17**, 109–949.
76 Oda, Y., Huang, K., Cross, F.R. *et al.* (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 6591–6596.
77 Pasa-Tolic, L., Jensen, P.K., Anderson, G.A. *et al.* (1999) *J. Am. Chem. Soc.*, **121**, 7949–7950.
78 Old, W.M., Meyer-Arendt, K., Aveline-Wolf, L. *et al.* (2005) *Mol. Cell Proteom.*, **4**, 1487–1502.
79 Ong, S.E., Blagoev, B., Kratchmarova, I. *et al.* (2002) *Mol. Cell Proteom.*, **15**, 376–386.
80 Schmidt, A., Kellermann, J., and Lottspeich, F. (2005) *Proteomics*, **5**, 4–15.
81 Ross, P.L., Huang, Y.N., Marchese, J.N. *et al.* (2004) *Mol. Cell Proteom.*, **3**, 1154–1169.
82 Thompson, A., Schafer, J., Kuhn, K. *et al.* (2003) *Anal. Chem.*, **75**, 1895–1904.
83 Chen, E.I. and Yates, J.R., III (2007) *Mol. Oncol.*, **1**, 144–159.
84 Veenstra, T.D. (2007) *J. Chromat.*, **B847**, 3–11.
85 Patel, V.J., Thalassinos, K., Slade, S.E. *et al.* (2009) *J. Proteom. Res.*, **8**, 3752–3759.
86 Mann, M. and Wilm, M.S. (1994) *Anal. Chem.*, **66**, 4390–4399.

16
Plant Tissue Culture and Genetic Transformation for Crop Improvement

Satbir S. Gosal and Manjit S. Kang

Plant tissue culture methods have a wide scope for the creation, conservation, and utilization of genetic variability for the improvement of field, fruit, vegetable, and forest crops and medicinal/aromatic plants. Micropropagation technology, particularly for vegetatively propagated plant species, ensures true-to-type, rapid, and mass multiplication of plants for quick bulking up of new varieties and rejuvenation of old varieties. Cellular techniques, such as anther/microspore culture, somaclonal variation, embryo culture, prototoplast culture, and somatic hybridization, are being exploited to generate useful genetic variability for incremental improvement of field crops. Using anther culture/pollen culture, several cultivars are either under tests or have been released in rice, wheat, barley, maize, rapeseed, and mustard in several countries. Furthermore, the doubled haploid approach is increasingly being used for the rapid development of populations for QTL mapping and construction of genetic linkage maps for traits of interest. Production of secondary metabolites, such as food flavors, food colors, dyes, perfumes, drugs, and scented oils used in aromatherapy, through cell cultures/hairy root cultures, are leading examples of molecular farming/pharming. Cryopreservation of germplasm at the cellular/tissue/organ level in liquid nitrogen at $-196\,^{\circ}C$ is highly rewarding for establishing germplasm banks, especially for vegetatively propagated crops and rare endangered plant species. Tissue culture-based genetic transformation has been commercially exploited for developing transgenic crop varieties in several crops.

16.1
Introduction

Plant tissue culture is a technique of growing plant cells, tissues, and organs in synthetic medium under closely controlled and aseptic conditions. It is based on the concept of totipotency, which refers to the capability of a cell to give rise to a complete plant under suitable cultural conditions. Such a property of cell has far-reaching implications in manipulation of plant cells for rapid multiplication of plants, to cross plants at the level of somatic cells by overcoming limits of crossability, and also to

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regenerate entire plants after genetic transformation. Suitable explants, that is, organs excised from plants, such as roots, hypocotyls, cotyledons, leaves, shoot apices, nodal segments, anthers, embryos, and seeds, are surface sterilized with a disinfectant like sodium hypochlorite (10–50% w/v for 10–30 min) or with mercuric chloride (0.1% w/v for 5–10 min), thoroughly washed with sterile water and then aseptically cultured in a synthetic medium in culture vessels such as test tubes, jars, and Petri dishes. The cultures incubated at $25 \pm 1\,^{\circ}C$ exhibit growth in 1–3 weeks depending upon the plant species, nature of explant, type of culture medium, kind and concentration of the growth regulators (hormones) used in the medium, and the light intensity in the incubation room.

Several media compositions have been developed for plant tissue culture, but the most commonly used media include [1–4]. Tissue culture medium contains major elements, microelements, vitamins, and amino acids, carbohydrates, and growth regulators (auxins, cytokinins, etc.). Auxins, such as indole acetic acid (IAA), indole butyric acid (IBA), and naphthalene acetic acid (NAA), at concentrations ranging from 0.1 to 5.0 $mg\,l^{-1}$, favor cell elongation and rooting, whereas 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 0.5–4.0 $mg\,l^{-1}$ usually induces callus, that is, homogeneous mass of undifferentiated cells. Likewise, cytokinins, such as 6-furfuryl amino purine (kinetin) and benzyl amino purine (BAP) at concentrations of 0.1–2.0 $mg\,l^{-1}$ cause rapid cell divisions and development of shoot buds/shoots. Solidification of the medium is achieved by adding chemically inert, powdered gelling agents, such as agar, agarose, and gelrite before autoclaving. The medium is poured into culture vessels and sterilized in an autoclave at 121 °C, 15 $lb\,inch^{-2}$ pressure for 20–25 min. Inoculation of explants in the culture vessels is done in Laminar Air Flow Cabinet fitted with HEPA filters (pore size 0.2–0.3 μm) under aseptic conditions. Placement of explants in suitable growth medium under appropriate conditions leads to dedifferentiation, that is, mature cells revert to meristematic state through enhanced DNA/RNA and protein synthesis. The renewed growth in agar-gelled medium gives an unorganized mass of cells, that is, callus. The cells may be cultured in liquid medium that gives a suspension of individual cells called suspension culture. An increased number of cells or calli leads to depletion of medium and thus the growing tissue needs to be transferred after every 3–4 weeks to fresh medium through subculturing. Ultimately, such cells/tissues are to be used to obtain organized structures such as roots, shoots, flower buds, and so on, through the process of organogenesis. Plant tissue and protoplast culture methods offer a rich scope for the creation, conservation, and utilization of genetic variability for the improvement of field crops [5, 6] (see Table 16.1). Various aspects of plant tissue culture in relation to crop improvement are given in Table 16.2.

16.2 Micropropagation

Micropropagation of plants is now one of the best and most successful examples of commercial application of tissue culture technology. Propagation of plants from very

Table 16.1 Plant tissue and protoplast culture techniques in relation to crop improvement.

Technique	Plant material	Remarks	References
Micropropagation	Banana *Musa* spp. (Philippine Lacatan and Grande Naine) plantain (Pelipita and Saba)	Rapidly multiplying cultures from excised shoot tips	[12, 13]
	Strawberry *Fragaria ananassa*	Efficient method for mass production of planting material	[21]
	Eucalyptus tereticornis	*In vitro* clonal propagation through nodal segments	[23]
	Rice *Oryza sativa* L. var. Jaya	Clonal propagation of indica rice through proliferation of axillary shoots	[34]
	Dalbergia sissoo Roxb.	Rapid *in vitro* propagation from mature trees	[24]
	Banana *Musa* spp cv. Grande Naine	Sunlight for micropropagation systems is a way of reducing tissue culture costs	[14]
	Potato *S. tuberosum*	*In vitro* propagation through microtubers	[177]
	Dianthus caryophyllus L. (carnation) cv. scania	*In vitro* propagation through axillary shoot proliferation	[25]
	Sugarcane *Saccharum officinarum* L.	Micropropagation protocol for mass plant production	[7–9, 11]
	Poplar *Populus deltoides*	Protocol for mass production of two important clones, namely, G_3 and G_{48} through induced shoot differentiation of leaf, stem, and root explants collected from adult trees	[26]
	Brahmi *Bacopa monnieri*	Efficient protocol for micropropagation through axillary shoot proliferation	[27]
	Rice *O. sativa* L. var. Mocoi FCA, Itapé P.A., Fortuna INTA, EMBRAPA7-Taim, and CT 6919 and BR IRGA 409	Clonal propagation protocol using shoot tip cultures, and the genetic stability of the micropropagated plants was verified by isozyme analysis	[35]
	Potato *S. tuberosum* L.	A protocol for production of prebasic potato material by microcuttings, obtained from plants with a short period of acclimatization	[18]
	Neem *A. indica*	Macro- and micropropagation protocols	[29]

(*Continued*)

Table 16.1 (*Continued*)

Technique	Plant material	Remarks	References
	Potato (*S. tuberosum* L.) Nif, Clone 122, Agria and Resy	The highest microtuber number (2.8), microtuber yield (278.1 mg) and single microtuber weight (92.2 mg) were obtained in the MS medium containing 2.0 mg BAP/l and 60 g sucrose	[178]
	Potato (*S. tuberosum* L.) cv. Marfona	The use of continuous and semicontinuous bioreactors and their functions at shoot multiplication and microtuberization of potato	[19]
	Banana *Musa sapientum*	Protocol for micropropagation of *M. sapientum* using shoot meristems	[15]
	Grapevine (*Vitis vinifera*)	Best shoot development for the initial culture of rootstock VR043-43 *in vitro* using nodal segments and best microcutting multiplication using QL medium	[30]
	Strawberry *F. ananassa*	Mass propagation via meristem tip culture	[22]
	Sugarcane *S. officinarum* L. cv. CoS 99 259	Spacing of 90 cm × 60 cm was most suitable for transplanting tissue-cultured plantlets	[179]
	Banana *Musa* spp.	Inflorescence apices were found more suitable for rapid *in vitro* propagation	[16]
	Grapevine (*V. vinifera*) cv. Bidaneh Sefid	Micropropagation protocol for quick multiplication	[180]
	Sugarcane *S. officinarum* L. cv. Co 86 032	Setts obtained from micropropagated plantlets resulted in higher seed yields	[181]
	Aloe vera	Micropropagated plants exhibited elevated levels of bioactive compounds	[32]
	Chrysanthemum cinerariifolium (Trev.)	Rapid propagation technology was established and optimized *in vitro*	[33]
	Banana *Musa* spp. cv. Cavendish Dwarf and Valery	Efficient medium for clonal mass propagation (MS + 30 gl^{-1} of sucrose, *N*-phenyl-*N*- 1,2,3-thidiazol 5-yl Urea (0.5 mgl^{-1}) and IAA (2 mgl^{-1}).	[17]
	Grape vine *V. vinifera* L. cv. Perlette	Clonal propagation of grapes for increasing plant material for cultivation	[31]
	Sugarcane *S. officinarum* L.	No variation was detected among the regenerated plants of a particular variety on the basis of RAPD markers, and the profiles of micropropagated clones were comparable to those of the respective donor plants	[182]
	Potato	Successful micropropagation was achieved using nodal segments as explants	[20]
	Scoparia dulcis	A suitable protocol was established through multiple shoot induction from nodal segment and shoot tip explants of this important medicinal herb	[183]

Meristem culture	*Chrysanthemum*	Production of virus free plants	[39]
	Sugarcane	Elimination of yellow leaf virus from infected sugarcane plants	[40]
	Sugarcane	Elimination of sugarcane mosaic virus using chemotherapy and meristem culture	[41]
	Bananas and plantains	Eradication of mosaic disease of bananas and plantains	[42]
	Banana	Eradication of banana bunchy top virus (BBTV) and banana mosaic virus (BMV) from diseased plants	[43]
	Potato	Production of virus-free plantlets	[44]
	Potato	Factors affecting *in vitro* growth of meristem tip-derived plantlets	[45]
	Red raspberry (*Rubus idaeus* L.)	Elimination of apple mosaic virus and raspberry bushy dwarf virus from infected plants	[46]
Somatic embryogenesis	Trifolium repens	Factors influencing coordinated behavior of cells as an embryogenic group	[47]
	Rice	Efficient plant regeneration from protoplasts through somatic embryogenesis	[48]
	Maize	Genotype specificity of somatic embryogenesis and plant regeneration	[53]
	Citrus (*Citrus reticulata* Blanco)	Factors enhancing somatic embryogenesis and plantlet regeneration in mandarin	[57, 58]
	Sugarcane	Factors enhancing somatic embryogenesis and plant regeneration	[51]
	Rice	Influence of antibiotic cefotaxime on somatic embryogenesis and plant regeneration	[49]
	Wheat	Ammonium nitrate improves direct somatic embryogenesis and biolistic transformation of *Triticum aestivum*	[50]
	Sugarcane	Desiccation of callus enhances somatic embryogenesis and subsequent shoot regeneration	[52]
	Maize	Moderate desiccation dramatically improves shoot regeneration from callus	[54]
	Cotton	Highly efficient plant regeneration through somatic embryogenesis in 20 elite commercial cultivars	[55, 56]
Somaclonal variation	*Prunus persica* cvs. Sunhigh, Red haven	Somaclones S156 and S122 resistant to leaf spot, moderately resistant to canker	[87]
	Apple rootstocks (M26, MM106)	S-2 (M26) performed better against *Phytophthora cactorum*	[88]
	Banana Giant Cavendish	Ten somaclones. GCTCV215-1 released for commercial planting	[184]
	Apple cv. Greensleeves	Sixteen somaclones; 21% less symptoms than Greensleeves against fire blight strain T.	[89]

(*Continued*)

Table 16.1 (*Continued*)

Technique	Plant material	Remarks	References
	Sugarcane *S. officinarum* L.	Results confirmed the superiority of two somaclones, one resistant and one tolerant to eyespot disease	[71]
	Potato *S. tuberosum* L.	Somaclones for heat tolerance	[77]
	Potato *S. tuberosum* L cv. Desiree	Somaclones IBP-10, IBP-27, and IBP-30, infected with *Alternaria solani* and *Streptomyces scabiei*, exhibited higher resistance to the pathogen, compared to the susceptible cultivar Desiree	[78]
	Potato cv. Desiree, tomato cv. Amalia leaves, soybean cv. William 82, coffee cv. Robusta	Monomorphism was detected in tomato, coffee, and soybean, indicating the genetic stability of the crops. Potato calli showed variations in the electrophoretic patterns of peroxidase and esterase isoenzymes, indicating somaclonal variation in the crop	[79]
	O. sativa L. cv. CICA-8	Four somaclones showed significantly higher degree of partial resistance compared to the parent cultivar CICA-8	[81]
	Durum wheat (*Triticum durum* Desf.) cv. (Selbera, Sebou, and Kyperounda)	Somaclonal variation thus appears to induce a wide range of modifications among individual components of drought-resistance mechanisms	[185]
	Sugarcane *S. officinarum* L. cv. CP-43/33	The somaclones performed better than the source plant	[73]
	Maize (*Zea mays*)	Somaclones thus derived were tolerant to NaCl	[80]
	Bread wheat (*Triticum aestivum*) cv. (Sakha 8, Sakha 69, Giza 157, Giza 160, Lerma Rojo 64, and Tobari 66)	Somaclones were superior to their original cultivars	[84]
	Wheat (*Triticum aestivum*) cv. Sakha 61	Twenty-one out of the twenty-three somaclones outperformed the original cultivar Sakha 61 in terms of leaf rust resistance and grain yield	[85]
	O. sativa L.	Somaclones were obtained from anther culture of hybrid combinations INCA LP-10/C4 153, Amistad-82/C4 153, and INCA LP-10, as well as from Amistad-82	[82]

	Sugarcane *S. officinarum* L.	Six tissue culture-derived sugarcane somaclones TC-434, TC-435, TC-436, TC-237, and TC-045 from CoC671 and somaclone number TC-338 from Co7219 were evaluated. Somaclone TC-435 gave higher cane yield at 12 mo crop age over CoC 671. Somaclone TC-435 had significantly higher millable cane height and number of internodes than that of the donor parent CoC671	[74]
	Sugarcane *S. officinarum* L.	A new sugarcane variety, Co 94 012, was released in the name of Phule Savitri for cultivation in Maharashtra, India, for Pre and Suru seasons. It is an early, sugar-rich, high-yield variety with high CCS yield. Co 94 012 is a somaclonal variant of CoC 671, with better sucrose content and moderate resistance to red rot (*Glomerella tucumanensis*) and smut diseases (*Ustilago scitaminea*). This is the first sugarcane variety to be released in India through the use of somaclonal variation	[10]
	Alfalfa *Medicago sativa*	An increase in variability was noted in important quantitative and qualitative traits compared to the initial cultivars, including productivity of the above-ground mass and seeds, resistance to fungal diseases, and winter hardiness	[86]
	O. sativa L. cv. Pokkali	The grain yield was significantly high in somaclones BTS 11-1, BTS 28, BTS 24, BTS 9-2(S), BTS-17(S), BTS 10-2, and BTS 11-7, which exhibited higher flag leaf area and moderate leaf area index compared to other somaclones	[83]
	Olive cv. Frangivento	Somaclonal variation could be found in olive plants regenerated through somatic embryogenesis; this appears in mature plants in the field	[186]
	Banana	In banana cultivars (*Musa* × *acuminata*, *Musa* × *balbisiana*), somaclonal variation can be useful in selecting clones with improved agronomic characteristics	[187, 188]
	Dieffenbachia cv. Camouflage, Camille, Star Bright	Potential for new cultivar development by selecting callus-derived somaclonal variants of *Dieffenbachia* was demonstrated	[189]
	Sugarcane *S. officinarum* L.	Development of somaclones resistant to red rot disease using *in vitro* and field selection	[75, 76]
In vitro production of haploid	Wheat	Doubled haploid wheat variety Florin was developed	[97]
	Wheat	A single 2,4-D treatment given to spikes one day after pollination with maize enabled embryos to be recovered from all 19 varieties	[98]

(*Continued*)

Table 16.1 (*Continued*)

Technique	Plant material	Remarks	References
	Rice	Revised medium was used for increasing anther culture efficacy and improved feasibility of using doubled haploids in genetic and breeding research with indica rice	[104]
	Rice	Bicol first F_1 anther culture- derived line from an indica/indica cross in saline-prone areas	[105]
	Rice	An improved method for pollen culture in rice	[106, 107]
	Durum wheat	Dicamba and 2,4-D was best for improving the yield of haploid plants of durum wheat through crosses with maize	[99]
	Maize *Z. mays* L.	Embryogenic induction of microspores within anthers under *in vitro* conditions was the best when a combination of cold treatment, TIBA (0.1 mg l^{-1}) in media and colchicine (0.02% during first 3 days of culture) was applied	[111]
	Citrus (*Citrus clementina*)	Anther culture as a rapid and attractive method of obtaining new triploid varieties in clementine	[110]
	Citrus (*C. clementina*)	Influence of light quality on anther culture of *C. clementina* Hort. ex Tan., cultivar Nules was reported	[111]
	Maize *Z. mays* L.	15, 10, 10, and 3 fertile doubled haploid plants were obtained in cultures treated with paraquat, *t*-BHP, methionine combined with riboflavin, and menadione, respectively	[102]
	Wheat	Simplified wheat × maize haploid production protocol that is 100% effective across all bread wheat cultivars, generating data means of 25% for embryo excision, 90–95% for plantlet regeneration, and between 95 and 100% for doubled haploid ($2n = 6x = 42$, AABBDD) outputs	[100]
	Maize *Z. mays* L.	Maize haploid plants by *in vitro* culture of pollinated ovaries	[128]
	Durum wheat *T. durum*	Novel pretreatment combining mannitol 0.3 M and cold for 7 days had a strong effect on the number of embryos produced and regenerated green plants. 11.55 green plants were produced per 100 000 microspores	[190]
	Tomato *Lycopersicon esculentum* L.	Embryogenesis and plant regeneration by *in vitro* culture of isolated microspores and whole anthers of tomato	[109]

	Rice	The 9 DH lines could provide the basic materials for breeding on Dian-type hybrid rice with both good quality and high blast resistance in the future	[108]
	Maize	Doubled haploids should be induced from F_2 plants rather than from F_1 plants	[103]
Embryo/ovule/ovary culture	*Vigna mungo* × *V. radiata*	Interspecific hybrids between *V. mungo* and *V. radiata* were produced through embryo culture	[143]
	Arachis	Embryo rescue from wide crosses in *Arachis*	[144]
	Moricandia arvensis × *Brassica*	lntergeneric (intersubtribe) hybrids between *M. arvensis* and *Brassica* A and B genome species by ovary culture	[140]
	V. vinifera L	Ovule culture of seedless grapes (*V. vinifera* L.)	[147]
	Brassica × *Sinapis*	Production of intergeneric hybrids between *Brassica* and *Sinapis* species by means of embryo rescue techniques	[141]
	Wheat × Rye	*In vitro* synthesis of white-grained primary hexaploid triticales	[142]
	Populus euphratica Oliv.	Intraspecific hybridization of *P. euphratica* Oliv. using *in vitro* technique	[146]
Protoplast culture and somatic hybridization	Potato *S. brevidens* × *S. tuberosum*	Somatic hybrids were produced by electrofusion	[191]
	Brassica, B. juncea, B. nigra, and *B. carinata* × *B. napus*	Resistance to *Phoma lingam* was expressed in all symmetric hybrids, and in 19 of 24 toxin-selected asymmetric hybrids	[152]
	Potato *S. brevidens* × *S. tuberosum*	Twenty hybrids tested expressed a high level of resistance to PVY	[150]
	Brassica, B. napus × *B. oleracea*	Inoculations *Xanthomonas campestris* pv *campestris* led to identification of four somatic hybrids with high resistance	
	Brassica, B. oleracea × *B. rapa*	Disease assays showed that most somatic hybrids had lower disease severity ratings against bacterial soft rot	[192]
	Citrus, *Citrus sinensis* L. Osbeck × *C. volkameriana Pasquale, C. reticulata Blanco*	Somatic hybrids combined characteristics from both sources and have potential for tolerance to blight and CTV	[153]
	Banana Maçã(*Musa* AAB group) × Lidi *Musa* sp. AA group.	Somatic hybrids were identified by using RAPD markers	[193]

(*Continued*)

Table 16.1 (*Continued*)

Technique	Plant material	Remarks	References
	Citrus mandarin × pummelo, sweet orange × pummelo	Somatic hybrids were confirmed by leaf morphology, ploidy analysis via flow cytometry, and RAPD analysis. Somatic hybrids have been propagated by tissue culture for further evaluation of disease resistance and horticultural performance in field trials	[194]
	Potato Aminca–Cardinal × Cardinal–Nicola	Complete resistance to PVY was noted for one somatic hybrid line (CN2). All other hybrids also showed improved tolerance to *Pythium aphanidermatum* infection during tuber storage or after plant inoculation	[151]
	Potato *S. tuberosum* × *S. tuberosum*	Tetraploid intraspecific somatic hybrids between 16 different diploid breeding lines of *S. tuberosum* L. were produced by PEG-induced fusion	[195]
	Brassica, B. oleracea × *B. rapa*	Calli were screened by RAPD analysis for their hybrid character. This is the first study reporting hybrid formation between two haploid protoplasts	[157]
	B. napus ($2n = 38$) × *Orychophragmus violaceus* ($2n = 24$) *B. napus* ($2n = 38$) × *O. violaceus* ($2n = 24$)	Symmetric fusions of mesophyll protoplasts and subsequent development of *B. napus–O. violaceous* chromosome addition lines	[196]
In vitro production of secondary metabolites	*Capsicum annum*	Accumulation of capsaicin in callus cultures	[159]
	Secondary metabolites, such as vincristine and vinblastine, and recombinant proteins	Plant suspension cells are an *in vitro* system that can be used for recombinant protein production	[160]
	Secondary metabolites	Hairy root culture for mass production of high-value secondary metabolites	[161]
Cryopreservation and *in vitro* germplasm storage	Rice *O. sativa* L.	Rice plants (cv. Taipei 309) were regenerated from different cryopreserved calli and cryopreservation was declared to be a reliable way to store transformation-competent rice lines	[166]

	Potato *S. tuberosum* L	Shoot tips of *in vitro* grown potato (*S. tuberosum* L.) plants were cryopreserved by a modified vitrification method	[167]
	Potato *S. tuberosum* L	*In vitro* plants of potato cultivars 'Superior' and 'Atlantic' were cold acclimated, and axillary buds were precultured, osmoprotected, exposed to PVS-2 solution, plunged into liquid nitrogen, thawed, and finally planted in the regeneration medium. After cryopreservation, vitrified shoot tips resumed growth within a week.	[168]
	Rubus spp. (blackberry and raspberry)	Encapsulation–dehydration and PVS2 vitrification cryopreservation protocols were found successful for preserving diverse *Rubus* germplasm (shoot tips)	[171]
	Arabidopsis	*Arabidopsis* can be successfully cryopreserved using either PVS2 or PVS3 as cryoprotectants prior to rapidly cooling shoot tips in LN	[164]
	Potato *S. tuberosum* L. cv. Dejima and (STN13)	Protocol was tested with 12 selected cultivated varieties and wild species, and the survival percentages obtained ranged between 64.0 and 94.4%	[169]
	Citrus	Up to 98.1% of the plants obtained by cryopreservation were free from HLB bacterium, compared to the sanitation rate of 25.3% yielded by conventional meristem tip culture	[170]
Genetic transformation	Direct gene transfer to plants	Particle mediated genetic transformation	[172]
	Rice	Analysis of transgene integration patterns, expression levels, and stability	[173]
	Various plant species	Various methods for genetic transformation and production of transgenic plants	[174]
	Agrobacterium-mediated transformation of basmati rice (*O. sativa* L.)	Expression of synthetic Cry1AB and Cry1AC genes for the control of European corn borer	[175]
	Transgenic crops	Global status of commercialized biotech/GM crops	[176]

Table 16.2 Various aspects of plant tissue culture.

S. No.	Aspect	Application
1	Micropropagation	True to type, rapid and mass multiplication of plants
2	Meristem culture	Production of disease-free plants
3	Somatic embryogenesis	Production of synthetic seeds and mass cloning of plants
4	Somaclonal variation	Induction of genetic variation particularly in vegetatively propagated species
5	*In vitro* production of haploids	Production of haploids/doubled haploids for early release of varieties
6	Embryo/ovule/ovary culture	Production of distant hybrids/alien gene transfer into cultivated varieties
7	Protoplast culture and somatic hybridization	Production of somatic hybrids and cybrids
8	*In vitro* production of secondary metabolites	Production of secondary metabolites (drugs, flavors, and dyes)
9	Cryopreservation and *in vitro* germplasm storage	Long-term storage of germplasm especially of vegetatively propagated species
10	Genetic transformation	Production of transgenic crop varieties/hybrids and molecular farming

small plant parts (0.2–10 mm) under *in vitro* conditions in the laboratories/polyhouses is called micropropagation. The technique of micropropagation was developed about 50 years back, but its commercial exploitation started only during the 1970s with orchids. Since then, it has seen tremendous expansion globally both in the number of production units and in the number of plants. It is estimated that more than 500 million plants of different plant species are now being produced annually through micropropagation in different parts of the world. Micropropagation industry is environment-friendly and requires little raw material in the form of chemicals. Because of higher labor costs in the developed countries, this industry is now being expanded in developing countries, where plant multiplication can be done at much cheaper rates for the international markets than in the developed countries.

16.2.1 Advantages of Micropropagation

1) It ensures true-to-type plants, that is, identical to mother plant (cloning).
2) Following micropropagation, selected plant species can be multiplied anywhere in the world.
3) It results in rapid and mass multiplication (1–10 per cycle of 2 weeks each) of elite clones/varieties that are otherwise difficult to multiply using conventional methods.
4) It is independent of seasonal and raw material constraints. Thus, the micropropagation industry can function throughout the year.

5) Micropropagation ensures disease-free plants and thus helps in rejuvenation of old clones or varieties.
6) It helps in the national and international exchange of germplasm, avoiding the risk of pathogens and insects.
7) Micropropagated, field-grown plants give higher yield and exhibit better quality.

16.2.2
Steps in Micropropagation

Step-1: **Establishment of Aseptic Cultures**
This is a transitory step, in which *in vivo*-grown plants are made to grow under *in vitro* conditions in the laboratory. Thus, it requires extra care and skill. Small plant parts, usually the shoot tips or nodal buds, are taken and disinfected with an appropriate disinfectant such as sodium hypochlorite or mercuric chloride for a specific period of time, depending upon the disinfectant used and the tissue being disinfected. Plant material is then cultured in a synthetic medium, under aseptic laboratory conditions. The cultures are kept in an incubation room maintained at $25 \pm 1\,^{\circ}C$, with a relative humidity of 60–80% and a light intensity of about 5000 lux, till new shoot buds/shoots appear (3–4 weeks).
Step-2: **Shoot Bud/Shoot Multiplication**
This is the real multiplication phase, in which cultures from the previous cycle are cut or divided and then recultured aseptically in fresh medium in separate culture vessels. This step involves cytokinins. During this step, there is nearly 10 times multiplication per cycle (2 weeks) throughout the year. Therefore, 26 cycles can be completed in a year, generating thousands of propagules. In this step, because of relatively high levels of cytokinins in the culture medium, the roots do not develop; hence, multiplication is generally in the form of shoot buds or shoots.
Step-3: **Induction of Roots and Hardening**
The shoots obtained from Step-2 are transferred to rooting medium to get the root primordia or root formation. Subsequently, hardening of plantlets is done by taking them out of the culture vessels and by washing them under slow-running tap water. The plantlets are then transferred onto water-moist cotton in trays and kept in the incubation room/polyhouse with daily change of tray water for a few days to get the plants acclimatized (Figure 16.1).
Step-4: **Transfer of Plantlets to Soil**
Hardened plantlets, individually or in clumps, are transferred to soil in polythene bags kept in the polyhouse for one and a half months before their delivery to end users.

16.2.3
Significance of Micropropagation

Micropropagation has special significance in the following areas:

1) Production of high-quality, disease-free, and superelite planting material for further seed production, especially in the vegetatively propagated plant species.

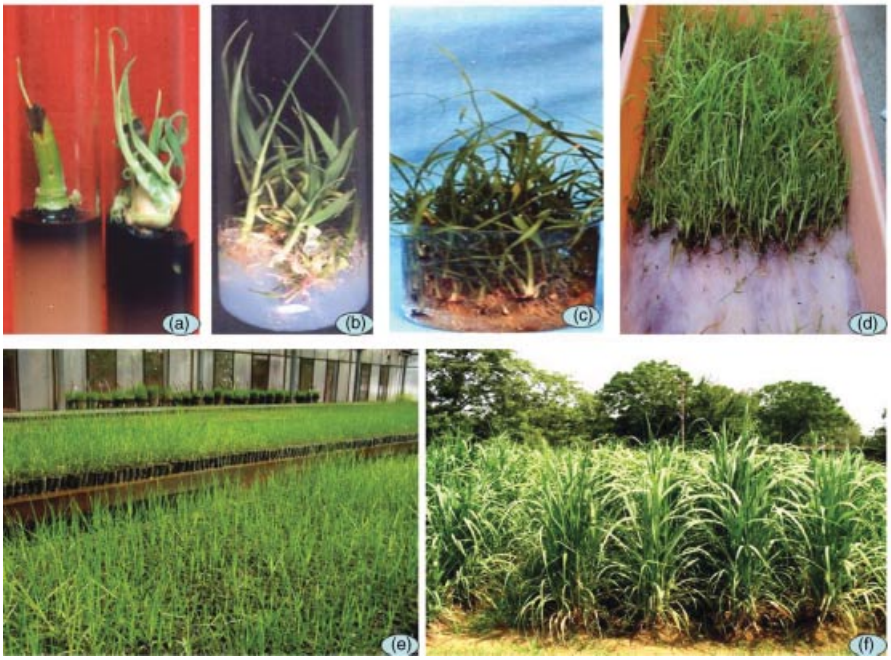

Figure 16.1 Micropropagation of sugarcane. (a) Establishment of shoot cultures *in vitro*. (b) Shoot multiplication. (c) Induction of rooting *in vitro*. (d) Hardening of plantlets. (e) Transfer of plantlets into soil in green house. (f) Micropropagated sugarcane in the field.

2) Rapid spread of new varieties of vegetatively propagated crops such as sugarcane, potato, poplar, and medicinal/aromatic plants for crop diversification.
3) Mass production of ornamental plants, which are otherwise difficult to multiply through conventional methods for domestic and international markets.
4) Rejuvenation of old varieties/clones of vegetatively propagated crops for improving their yield and quality.
5) Mass cloning of rootstocks in horticultural plants such as citrus, peach, and apple.
6) Mass cloning of genetically plus, cross-pollinated, and seed propagated trees.
7) Multiplication of male sterile lines for hybrid seed production or the multiplication of F_1 hybrids and transgenic clones/varieties.
8) During genetic transformation, this technique helps in increasing the plant number of elite events for precise characterization and efficient transfer of regenerated plants to greenhouse.
9) During anther/pollen culture, micropropagation of regenerants helps in minimizing the risk of losing any genotype during their hardening and transfer to soil.

10) Interstate/international exchange of germplasm, avoiding the risk of pathogens and insects.
11) It possesses tremendous potential in keeping our environment clean and green.

Now scores of multimillion-dollar industries around the world propagate a variety of plant species through tissue culture, which allows environment-friendly industries to flourish. The clean planting material can certainly improve the yield potentials of vegetatively propagated crops such as sugarcane, potato, banana, strawberry, sweet potato, cassava, and several ornamental plant species. Micropropropagation protocols for some important crop plants have been developed and are being exploited for commercial plant production. Some of these include sugarcane [7–11], banana [12–17], potato [18–20], strawberry [21, 22], eucalyptus [23], *Dalbergia sissoo* [24], carnation [25], poplar [26], brahmi (*Bacopa monnieri*) [27, 28], neem (*Azadirachta indica*) [29], grapevine [30, 31], Aloe vera [32], *Chrysanthemum* [33], and rice [34, 35]. It is likely that automation of multiplication systems will be commercially feasible within the next few years.

16.3 Meristem Culture

Virus infection in plants reduces both yield and quality. Replacement of virus-infected stock with healthy stock (virus free) has shown up to 300% yield increase [36, 37]. In most of the seed-propagated crops, the gametes serve as sieve against a variety of pathogens and help in production of disease-free seeds, whereas in vegetatively propagated species, the pathogens keep on accumulating generation after generation, which ultimately cause varietal decline. There are no effective chemical methods to control viral diseases. In this regard, meristem culture [38] is a practical approach for producing disease-free plants. Shoot meristems (0.2–0.4 mm) excised from *in vivo*- or *in vitro*-grown plants are aseptically cultured *in vitro* in culture medium under suitable cultural conditions. Meristem-derived plants are screened by using electron microscopy/immunological methods for selecting pathogen-free plants [39]. Disease-free plants are then micropropagated for production of superelite planting material. This technique is well established for the production of disease-free planting material of sugarcane [40, 41], bananas and plantains [42, 43], potato [44, 45], and apple [46].

16.4 Somatic Embryogenesis

Somatic embryogenesis is the process by which somatic cells develop into differentiated plants through characteristic embryological stages without the fusion of gametes [47]. A number of factors, such as genotype of donor plant, medium constitution, auxins, sugars, amino acids, growth retardants, desiccation, and so on, influence the process of somatic embryogenesis and subsequent plant regen-

eration. Embryogenic callus/cell suspension cultures ensure high-frequency plant regeneration upon their transfer to shoot regeneration medium. Therefore, such cultures are preferred for genetic transformation. Furthermore, somatic embryos can be encapsulated in a suitable matrix, containing nutrients, growth regulators, antibiotics, and so on, needed for the development of a complete plant to make what are known as synthetic or artificial seeds that can be stored for several years and can be sown like natural seeds. Frequent somatic embryogenesis has been reported in rice [48, 49], wheat [47, 50], sugarcane [51, 52], maize [53, 54], cotton [55, 56], and citrus [57, 58]. Improvement of somatic embryogenesis [51], coupled with embryo desiccation and encapsulation technology, may lead to the utilization of artificial seeds for mass cloning of plants.

16.5 Somaclonal Variation

Variation among tissues or plants derived from the *in vitro* somatic cell cultures, that is, callus and suspension cultures, is called somaclonal variation. It may be genetic or may result from culture-induced epigenetic changes [59]. The epigenetic changes are expressed at cell culture stage, but these changes usually disappear when plants are regenerated or reproduced sexually. Variation arising out of anther/pollen culture is more precisely known as gametoclonal variation and that through protoplast culture is called protoclonal variation. It, therefore, provides a novel mechanism to generate new genetic variation for crop improvement.

16.5.1 Induction of Somaclonal Variation

Callus cultures are established from suitable explants and multiplied through periodic subculturing (Figure 16.2). Likewise, cell suspension cultures can be established by transferring actively growing callus to constantly agitated liquid medium and can be maintained/multiplied through periodic subculturing. Plants are regenerated usually from long-term maintained (old) callus/cell suspension cultures and transferred to soil and screened for variation, in the glasshouse or field. *In vitro* selection at cellular level can be carried out for some traits by growing cells from cell suspensions and calli on a medium supplemented with elevated levels of various biotic and abiotic stress factors (only the variant cells survive). Using this technique, many million cells (potential plants) can be screened in a single Petri dish, which is practically difficult, if not impossible, to be adapted at whole-plant level in the field. Moreover, *in vitro* selection also reduces the chances of diplontic selection, but it requires high level of correspondence between the trait(s) selected *in vitro* and expressed *in vivo*. Somaclonal variants can be identified through screening, which involves assessment of regenerated plants for characters such as yield that cannot be evaluated at single-cell level or through cell selection that involves application of suitable selection pressure like that of some toxins to permit preferential survival of variant cells.

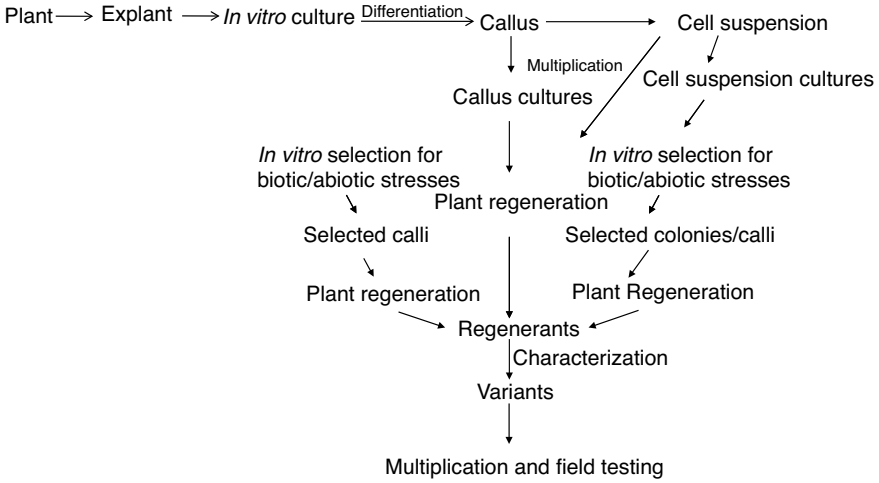

Figure 16.2 Scheme for induction and selection of somaclonal variation in crop plants.

16.5.2
Causes of Somaclonal Variation

Somaclonal variation may be genetic or epigenetic; the genetic variation is heritable, whereas the epigenetic variation, caused by cultural conditions, is not heritable and hence has no significance in sexually propagated plants. Genetic variation may result from the following causes:

- **Chromosomal Changes**
  Chromosomal changes in tissue culture-derived plants have been observed with respect to both chromosome number and structure. Besides polyploidy, aneuploidy (monosomics and trisomics) has been observed in oats, ryegrass, wheat, triticale, and potato. There have been a number of studies of modified chromosome structure in cultured plant cells [60]. Deletions, inversions, translocations, and duplications have been frequently observed in barley, wheat, potato, and maize. While large changes in chromosome structure have been detected, it is likely that less dramatic structural changes that were not detected occur quite frequently. Small changes in chromosome structure could alter expression and genetic transmission of specific genes. In addition, recombination or chromosome breakage may occur in preferential regions or "hot spots" of particular chromosomes, thereby affecting some regions of the genome in a disproportionally higher frequency [61].
- **Mitotic Crossing Over**
  Mitotic crossing over may account for some of the genetic variation that leads to the recovery of homozygous recessive single-gene mutations in some regenerated plants. Dulieu and Barbier [62] regenerated plants from *Nicotiana tabacum* with specific chlorophyll deficiency markers present in heterozygous condition. A high

frequency (9.6%) of variant regenerants at the "a_1" and "y" loci has been ascribed to the combination of deletion and mitotic recombination.

- **Apparent "Point" Mutations**
  Genetic changes resembling single-gene mutations have now been detected in numerous crops. The recessive single-gene mutations are suspected if variant does not express itself in the regenerant (R_0) plant, but the self-fertilized R_1 progeny segregates in an expected 3 : 1 Mendelian ratio for a morphological trait. This type of analysis has been completed for several tomato somaclones and used to map somaclones to specific loci [63]. Such a phenomenon has also been noted in maize, tobacco, rice, and wheat.
- **Cytoplasmic Genetic Changes**
  Cytoplasmic genetic changes involving mitochondrial DNA (mtDNA) have been described in maize by evaluating plants for two cytoplasmic traits [64]. Sensitivity to host-specific toxin of *Drechslera maydis* race T, the causal agent of southern corn leaf blight, is associated with all genotypes containing Texas male sterile (cms-T) cytoplasm. Such traits are tightly linked and controlled by mitochondrial DNA. Gengenbach *et al*. [64] selected *in vitro* for resistance to toxin and regenerated resistant plants with the aim of recovering toxin-resistant cytoplasmic male sterile lines, but among the regenerants, resistance was associated with reversion to male fertility. Restriction endonuclease pattern of mtDNA revealed significant changes in mtDNA. This mutation to male fertility and toxin insensitivity has been shown to be a frameshift mutation in mitochondrial DNA.
- **Deamplifications and Amplifications**
  Deficiencies in ribosomal DNA (rDNA deamplifications), although not associated with change in plant morphology, have been observed at the molecular level in flax, triticale, and potato. On the other hand, gene amplifications, that is, duplications, have been observed in *Nicotiana* [65] and tomato.
- **Transposable Element Activation**
  Activation of otherwise silent controlling elements (mutator genes) has been observed following plant cell cultures of *Nicotiana*, alfalfa, and maize. Chromosome breakage and fusion, which occur in culture, and genomic stress caused by culture conditions are major causes of transposable element activation. Transposable elements are known to cause phenotypic changes in plants and their activation during *in vitro* culture induces somaclonal variation [66].
- **Virus Elimination**
  Virus infection in several instances causes changes in the plant's reaction to other diseases. For example, prior infection with barley yellow dwarf virus causes susceptibility to powdery mildew in oats. Larkin and Scowcroft [59] suggested that the virus elimination during *in vitro* passage could alter the plant's susceptibility to certain fungal diseases and the somaclones exhibited resistance.
- **Methylation/Demethylation of DNA**
  De novo methylation and demethylation events are part of differential genomic changes. Tissue-specific DNA methylation of different sequences has been reported for several plants. Genome activity, that is, transcription, replication,

rearrangement, and the structural organization of chromatin, somehow seems to be related to DNA methylation [67, 68].

- **Altered Expression of Multigene Families**
  It has been postulated that the cultural conditions may regulate the expression of the multigene family in a way that a member gene that previously expressed some agronomically important genes, including those for gliadins, zeins, glutenins, α-amylase, are coded on multigene families. Heritable somaclonal variation has been obtained for gliadin – a storage protein – and β-amylases in wheat [69].

16.5.3
Nature of Somaclonal Variation

Somaclonal variation has been reported in several crops for both qualitative and quantitative traits, including male sterility in maize; improved protein content in rice and triticale; high sucrose content in sugarcane; early tasseling in corn; changed plant height, awns, tiller number, grain color, heading date, gliadin proteins, and α-amylase in wheat; herbicide tolerance in tomato; disease resistance in maize, sugarcane, mustard, and potato; and salt tolerance in rice [70]. However, such somaclonal variations have not been frequently utilized because, in many cases, these include either the already existing types or there were desirable changes accompanied by several undesirable changes.

16.5.4
Significance of Somaclonal Variation in Crop Improvement

Several interesting and potentially useful traits have been recovered using this method in sugarcane [10, 71–76], potato [77–79], maize [80], rice [81–83], wheat [84, 85], alfalfa [86], *Prunus persica* [87], and apple rootstocks [88, 89]. Recovery of novel variants that either do not exist or are rare in the natural gene pool, for example, atrazine resistance in maize, glyphosate resistance in tobacco, improved lysine and methionine contents in cereals, increased seedling vigor in lettuce, jointless pedicels in tomato, and *Fusarium* resistance in alfalfa, are of much significance [63, 76]. Genetic, cytogenetic, and molecular evidence for increased recombination frequency through cell culture has now been provided [90]. Tissue culturing of wide hybrids also helps in breaking undesirable linkages and achieving introgression from alien sources. Several new varieties have been developed through somaclonal variation in tomato, sugarcane, potato, celery, *Brassica*, and sorghum. This simple and cost-effective technique has a huge potential for the improvement of apomictic and vegetatively propagated plant species and, of course, seed-propagated crop plants with a narrow genetic base. In India, a somaclonal variant of a medicinal plant, *Citronella java*, has been released as a commercial variety, B-3, which gives higher yield and oil content than original variety. Likewise, Pusa Jai Kishan is a variety of *B. juncea* that has been released as a somaclonal variant of Varuna variety. However, in several situations, low plant regeneration ability and lack of

correspondence in expression of the trait in field-grown plants are the major problems [91].

16.6 *In Vitro* Production of Haploids

In self-pollinated crops, an inordinately long period is required to assemble desirable gene combinations from different sources in homozygous form. Generally, it takes 8–10 years to develop stable, homozygous, and ready-to-use materials from a fresh cross of two or more parental lines. In cross-pollinated crops, because of inbreeding depression, it becomes difficult to develop vigorous inbreds for hybrid seed production programs. In this regard, haploids possessing gametic chromosome number are very useful for producing instant homozygous true-breeding lines. In addition, haploids constitute an important material for induction and selection of mutants, particularly for recessive genes. In conventional breeding, the early-segregating generation populations involve variation attributable to both additive and nonadditive genetic effects [92], whereas doubled haploid (DH) lines exhibit variation only of additive genetic nature, including additive × additive type of epistatis, which can be easily fixed through a single cycle of selection. The elimination of dominance effects leads to high narrow-sense heritability, and availability of sufficient seed of each DH line allows for replicated testing. Thus, in contrast to relatively large segregating populations in conventional genetic studies, fewer DH lines are required for the purpose of selection of desired recombinants. For instance, in rice, about 150 DH lines derived from F_1, instead of 4000–5000 F_2 plants, are sufficient for selecting desirable genotypes. Production of haploids has also been exploited during wide hybridization for the development of addition and substitution lines. Production of haploids/doubled haploids through anther culture from F_1 rice plants results in true-breeding plants in less than 1 year, which otherwise takes 7–8 generations in conventional methods [93] (Figure 16.3).

16.6.1 Methods of Haploid Production

Four important methods for *in vitro* production of haploids are as follows:

i) Anther culture
ii) Isolated microspore culture
iii) Unpollinated ovary culture
iv) Embryo rescue from wide crosses

16.6.1.1 Anther Culture

Anther/pollen culture is an attractive alternative for developing haploids (sporophytes with gametophytic chromosome number). One of the very popular methods for production of haploids is anther or microspore culture. Incubation of cultures under optimum conditions leads to growth of microspores into sporophytes. The

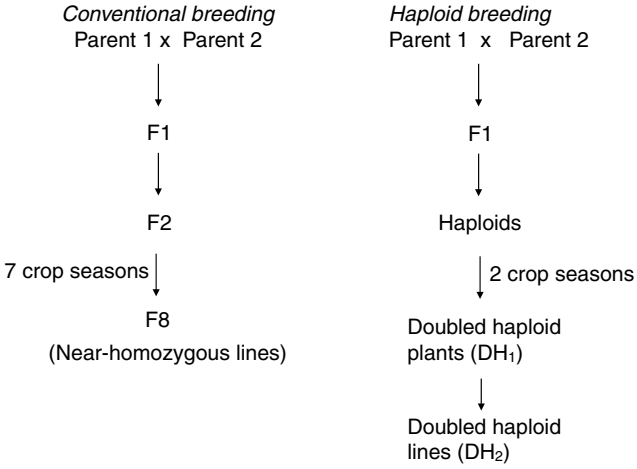

Figure 16.3 Period required to develop homozygous lines through conventional and haploid breeding.

following parameters have been recognized as particularly important for successful anther/microspore culture: (i) growth conditions of donor plant, (ii) genotype of donor plant, (iii) pretreatment of anthers, (iv) developmental stage of anthers/microspores, (v) composition of culture medium, and (vi) physical conditions during culture growth. Anthers are cultured in liquid or on semisolid agar medium [94], where they may directly give rise to embryoids or may lead to callus formation before differentiation. The embryoids develop into haploid plantlets or doubled haploids in some crops (because of spontaneous doubling of chromosomes during callus proliferation). Haploids may be treated with colchicine to obtain fertile, doubled haploid homozygous plants for field testing and selection. The technique of anther culture was first developed in *Datura* by Guha and Maheshwari [95, 96]. Flower buds at an appropriate stage are collected from healthy greenhouse- or field-grown plants. The collected flower buds are usually wrapped in plastic bags and kept in a refrigerator at 4 °C for 7–10 days for cold treatment. Flower buds are surface sterilized with 0.1% $HgCl_2$ for 9–10 min and anthers are carefully dissected out of the flower buds and inoculated in the medium. Cultures are incubated at 25 ± 1 °C under diffused light conditions. In general, the cultured anthers exhibit callusing after 2–6 weeks. About 1 month-old calli are made to regenerate into plants (Figure 16.4).

16.6.1.1.1 **Significance of Anther Culture** Anther culture systems (see Figure 16.5) have been developed for several important crop plants, such as wheat [97–100], maize [101–103], rice [104–108], tomato [109], and citrus [110, 111]. Several cultivars are either under test or have been released for rice, wheat, maize, rapeseed, and mustard in China, Canada, Denmark, the United States, and France [112, 113]. Furthermore, the doubled haploid approach is increasingly being used for rapid development of populations for QTL mapping and construction of genetic linkage maps for traits of interest.

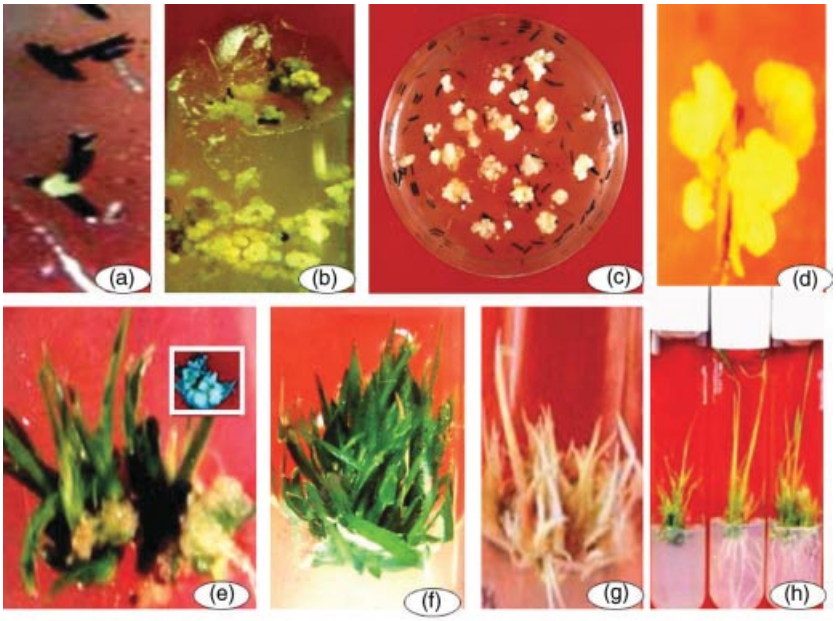

Figure 16.4 Anther culture in indica rice. (a) Cultured anthers showing callus initiation on the culture medium. (b) Callus formation from rice anthers cultured in test tube. (c) Callus formation from rice anthers cultured in the Petri plate. (d) Callus proliferation from cultured anther as seen under stereomicroscope. (e) Shoot regeneration from anther-derived callus. (f) Shoot proliferation. (g) Regeneration of albino shoots from anther-derived callus. (h) Complete plantlets obtained from anther-derived callus.

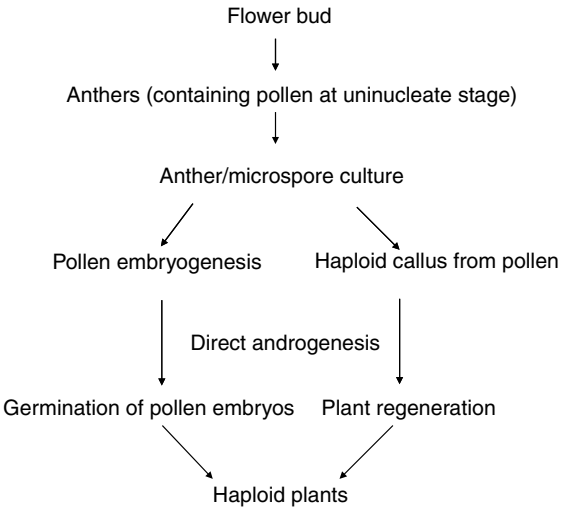

Figure 16.5 Schematic representation of production of haploids through anther/microspore culture.

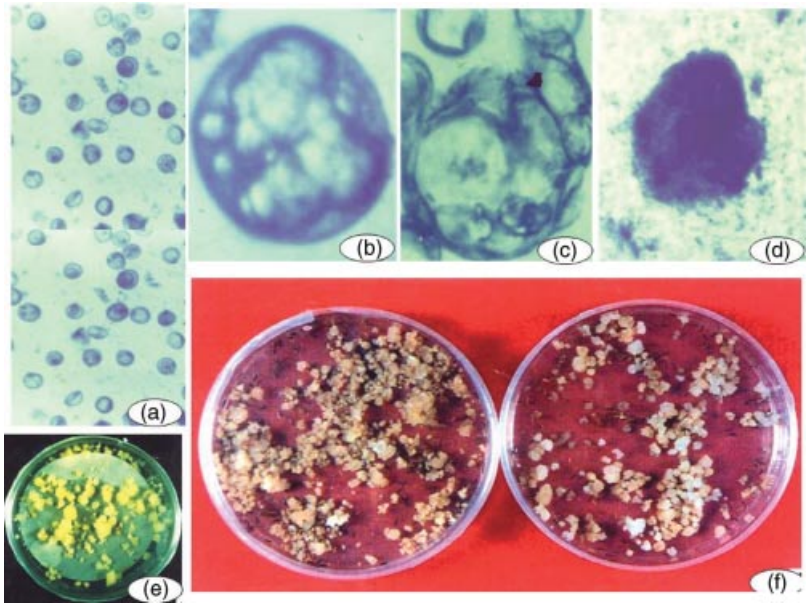

Figure 16.6 Pollen culture in indica rice. (a) Pollen cultured in liquid medium. (b) Segmentation in pollen cytoplasm. (c) Pollen embryoid. (d) Fully developed pollen embryo. (e) Pollen-derived callli on filter membrane. (f) Calli obtained from pollen embedded in agar solidified medium.

16.6.1.2 **Isolated Microspore Culture**

Microspore culture [114] (Figure 16.6) has several advantages over anther culture because microspores are haploid single cells that can readily be genetically manipulated. Unlike anther culture, microspore culture eliminates the participation of diploid tissues (anther wall and connective tissues). The two methods of pollen isolation are (a) naturally shed pollen in the culture medium after a preculture of anthers and (b) mechanical means by crushing or magnetic stirring. The naturally shed pollen grains are known to result in more calli and plantlets than mechanically isolated pollen of rice, barley, and tobacco. In case of rice, large microspores (50–58 μm) with thin pink colored outer walls produced embryos, whereas the division of small (40 μm) microspores with thick cell walls was not observed [115]. Addition of glutamine, proline at 1 mM concentration, and ficoll 10% (w/v) into the culture medium has shown beneficial effects during the isolated microspore culture in the liquid medium. Most of the factors affecting anther culture also affect the success in pollen culture. Microspore/pollen culture has been an attractive alternative for haploid production in *Brassica napus* [116–119], *B. juncea* [120–123], *Hordeum vulgare* [124], wheat [125], oats [126], and pepper [127].

16.6.1.3 **Ovary Culture**

Induction of haploids from megaspores, also known as gynogenesis, was reported by culturing unpollinated ovaries of *Zea mays* [128]. Subsequently, the technique

has also been extended to other crops, including barley and rice. Genotypic differences have been observed for the development of gynogenic calli. The rate of success also varies with species and is strongly influenced by the genotype. A japonica type of rice genotype is far more responsive than varieties of indica types. The success of ovary culture mainly depends on the development stage of the ovary. Success has been reported with ovaries ranging from uninucleate to mature embryo-sac stages. Regulation of growth regulators to enhance gynogenesis, but to inhibit proliferation of somatic tissues, has been very critical for ovary culture [129]. Rice ovaries failed to enlarge in the absence of MCPA (2-methyl, 4-chlorophenoxyacetic acid). An increase in MCPA concentration from 0.125 to 8 mg l^{-1} favored ovary swelling. In contrast to anther culture, ovary culture is inefficient because there is only one embryo sac per ovary compared to thousands of microspores per anther. However, the rate of induced embryo sacs has generally been higher than for microspores and the frequency of green-plant regeneration has also been higher than from anther cultures. But ovary culture has been successful only in a few species.

16.6.1.4 **Embryo Rescue for Wide Crosses**

Embryo rescuing from wide crosses in some crops serves as an alternative route to haploidy. Moreover, the system is less prone to gametoclonal variation owing to the absence of redifferentiated callus phase. The phenomenon is based on the elimination of a full set of chromosomes of one of the parents during *in vitro* embryo development.

16.6.1.4.1 **Bulbosum Method** This method was first developed for production of haploids in diploid barley by Kasha and Kao [130] (Figure 16.7). The haploids are produced from interspecific crosses between *Hordeum vulgare* (female) and

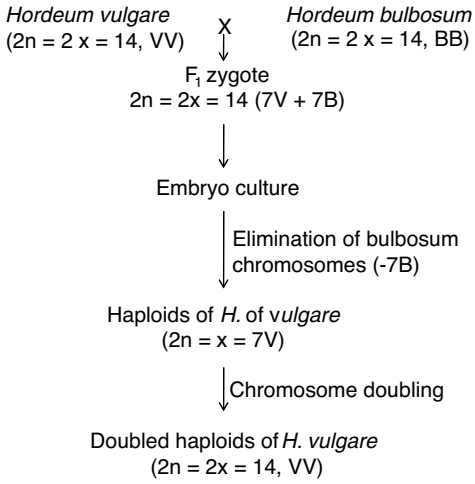

Figure 16.7 Schematic representation of bulbosum method.

H. bulbosum (male). Zygote induction is fairly high and the chromosomes of *H. bulbosum* are rapidly eliminated from the developing embryos. Developing endosperm also aborts after about 2–5 days of growth, which necessitates the rescuing of embryos in order to complete their development. Embryo culture using nutritionally rich medium results in complete haploid plants of *H. vulgare*, and chromosome doubling is induced in the established plants. Barclay [131] extended this method to wheat, in which haploids of wheat were obtained through embryo culture from *Triticum aestivum* cv. Chinese Spring × *H. bulbosum* cross. However, this method is restricted to wheat varieties possessing "kr" crossability genes that are responsible for the elimination of *H. bulbosum* chromosomes.

16.6.1.4.2 **Wheat Haploids from Wheat × Maize Crosses** Zenkteler and Nitzsche [132, 133] were the first to report microscopic early-stage embryos in crosses between wheat and maize. Subsequently, Laurie and Bennet [134, 135] demonstrated the presence of both wheat and maize chromosomes in zygotes and found that maize chromosomes were eliminated during initial cell divisions. Endosperm development ceases early or never occurs, and embryos fail to develop to a size that can be readily rescued. Complete wheat haploid plants using wheat × maize system by employing *in vitro* culture of wheat spikelets, 2 days after pollination, were obtained [98]. This method has been successfully extended to durum wheat [136] and to field-grown bread wheat by daily injecting 2,4-D (125 ppm) into pollinated tillers for 3 days, followed by embryo culturing 15 days after pollination with maize [137, 138].

Haploids produced through any of these methods have been used to produce doubled haploid lines through colchicine treatment. The doubled haploids produced from F_1 plants represent a set of new recombinant lines in homozygous state, which can be used either for commercial cultivation or for further breeding purposes.

16.7 Embryo/Ovule/Ovary Culture for Wide Hybridization

During wide hybridization, when parents are genetically diverse, endosperm degeneration leads to embryo abortion and the failure of the cross. Under such situations, after making desired pollination, developing embryos, ovules, and even ovaries are aseptically excised and cultured *in vitro* using suitable culture medium and physical cultural conditions. The plantlets thus obtained are transferred to greenhouse and characterized using morphological, cytogenetic, biochemical, and molecular approaches. In case of seed sterility, the plantlets are treated with colchicine for production of amphiploids through chromosome doubling. It has been a practical approach [139] to obtain interspecific and intergeneric hybrids in *Brassica* [140, 141], wheat [142], *Vigna* [143], *Arachis* [144], *Lilium* [145], and *Populus* [146]. Ovule culture in grapes has been attempted for developing hybrids, even in seedless grapes [147]. These methods have been successfully used to transfer desirable genes from wild relatives into cultivated varieties of several field and vegetable crops.

16.8
Protoplast Culture and Somatic Hybridization

Somatic hybridization is an effective approach to hybridize sexually incompatible species. Complete fusion of nuclei and cytoplasms of somatic cells from both species leads to formation of somatic hybrid cell and plant. Likewise, the fusion of cytoplasm from two species and nuclear genes from any one leads to the development of a cybrid. The plant cells are surrounded by a thick cell wall that does not allow cells to fuse to get somatic hybrid cell/plant. However, protoplasts can be easily fused and employed in several other experiments aimed at the genetic modification of plants (Figure 16.8). A protoplast is a naked cell without cell wall, surrounded by plasma membrane, and potentially capable of cell wall regeneration, growth, and division. The techniques of isolation, culture, and regeneration of protoplasts have been established in more than 100 plant species, including major field, vegetable, and fruit crops. Protoplast technology (see Figure 16.9) basically involves five steps: (1) isolation of protoplasts, (2) fusion of protoplasts, (3) culturing of protoplasts, (4) regeneration of plants, and (5) characterization of protoplast-derived plants.

Since the production of the first somatic hybrid between *N. glauca* and *N. langsdorfii* in 1972, numerous intraspecific, interspecific, and intergeneric hybrids have been produced. Somatic hybrids fall into two categories, namely, symmetric and asymmetric [148]. Symmetric hybrids consist of complete sets of chromosomes from both the parents, whereas asymmetric hybrids possess full chromosome complement of only one parent. Earlier, efforts were made to obtain somatic hybrids among closely related and cross-compatible species, where somatic hybrids resembled the sexual

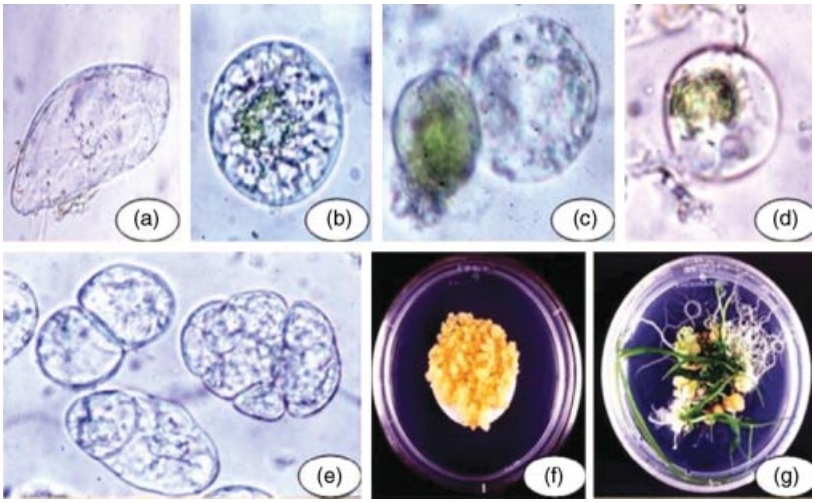

Figure 16.8 Protoplast culture in rice. (a) Somatic cell from cell suspension culture of rice. (b) Isolated protoplast from cell suspension culture. (c) Protoplast aggregation during fusion. (d) Fused protoplasts. (e) Dividing protoplasts during culture. (f) Protoplast-derived colonies on filter membrane. (g) Plant regeneration from protoplast-derived colonies.

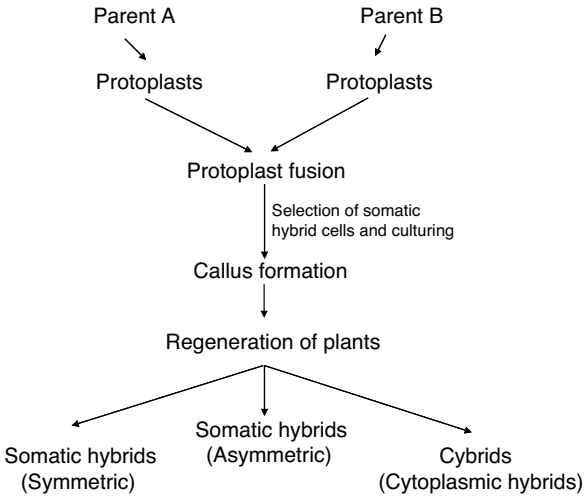

Figure 16.9 Scheme for production of somatic hybrid/cybrid plants.

hybrids. For instance, the somatic hybrid between *B. campestris* and *B. oleracea* resembled *B. napus*. With refinements in the techniques of protoplast isolation, fusion, and culture, people thought to produce novel hybrids by fusing protoplasts from remote species. However, somatic incompatibility has been observed to operate at various levels and different growth patterns have been observed; for example, hybrid cells underwent only few divisions, fusion products grew successfully as undifferentiated cells, and morphogenesis resulted in teratomas (highly abnormal and sterile plants). Asymmetric hybrids carry partial genomes from the donor species. Asymmetrization occurs spontaneously or it can be induced artificially. The final product of protoplast fusion among phylogenetically remote species is usually an asymmetric combination of two genomes, with parts of one or both genomes being lost during the *in vitro* passage. The extent and direction of asymmetrization is largely random, hence unpredictable. Asymmetrization is essential for improving plant regeneration, but it is random and gradual; hence, it may not be potentially desirable at least for the applied objectives. Like nuclear genes, asymmetrization also occurs in cytoplasmic genes. Now, there is growing interest in artificial production of asymmetric cytoplasmic hybrids (cybrids) for a single-step transfer of useful cytoplasmic traits such as male sterility, disease resistance, and herbicide resistance. The protoplasts of the donor species are X-irradiated (9–50 kr) or treated with iodoacetate to inactivate the nuclear genome. In addition, miniprotoplasts (protoplasts lacking nucleus) from donor species are also being increasingly used. In the case of somatic hybrids combining full nuclear genomes, the parental genomes usually remain spatially separated within the nucleus. Effects of spindle-disturbing chemicals such as colchicine, chlorosopropyl-*N*- phenyl-carbamate, and aminoprophosmethyl (APH) are being investigated to induce translocations/recombinations.

16.9
Significance in Crop Improvement

Protoplast culture and somatic cell hybridization, involving fusion of protoplasts from different plant species, are important approaches for combining characteristics even from otherwise sexually incompatible species. Furthermore, cybrids and organelle recombinants, not possible with conventional methods, can also be developed [148]. Therefore, researchers' interest has moved from creation of novel hybrids to the production of cybrids, chromosome transfer, and gene introgression [149]. It is well known that alloplasmic association leads to male sterility as a consequence of interactions between nuclear and mitochondrial elements. Male sterility has been developed by fusing protoplasts of *N. tabacum* with X-irradiated protoplasts of *N. africana*. Likewise, male sterility has been transferred from *Raphanus sativus* into *B. napus*. Moreover, resistance to triazine herbicide has been combined with male sterility by fusing *B. napus* protoplasts from a male-sterile line with *B. napus* protoplasts from a triazine-resistant parent. Cytoplasmic genetic male sterility has also been successfully transferred into rice. Resistances to some diseases such as potato leaf roll virus, PVX, and PVY have been incorporated into *Solanum tuberosum* from *S. brevidens* and *S. phureja* through protoplast fusion [150, 151]. Likewise, resistance against Phoma lingam disease in *Brassica* species [152] and citrus tristeza virus (CTV) in citrus [153] was developed through protoplast fusion. Following protoplast fusion, hybrids of Iris fulva (4x) × Iris laevigata were produced. Iris fulva has unique brown flowers, and this trait could be very useful for flower color improvement in Iris laevigata, which lacks this color [154]. Protoplast-to-plant system developed in basmati rice [155, 156] can be exploited for single-step transfer of male sterility from one line to another for the production of hybrid rice. Pollen protoplasts have also been fused using pollen protoplasts of *B. oleracea* var. italica and haploid mesophyll protoplasts of *B. rapa* [157].

16.10
In Vitro Production of Secondary Metabolites

Several plant species are known to produce secondary metabolites *in vivo* or *in vitro*. These metabolites do not perform vital physiological functions, but some act as potential predators and attract pollinators. Furthermore, these metabolites act as a valuable source of a vast array of chemical compounds, including fragrances, flavors, natural sweeteners, and industrial feedstocks. Cultured cells/organs produce a wide range of secondary products. Mainly three approaches have been followed: (i) the rapid growth of cell suspension cultures in large volumes, (ii) immobilization of plant cells, and (iii) growing hairy root cultures *in vitro*. There are several advantages of the cell culture systems over the conventional cultivation for the production of secondary metabolites, for example, (1) independence from various environmental factors, (2) any plant cell can be multiplied to yield specific metabolite, and (3) culture of cells may prove suitable where plants are difficult or expensive to grow in the field

because of their long life cycles. Since the 1970s, when the possibility of producing useful secondary products in plant cell cultures was first recognized, considerable progress has been made and a number of plant species have been found to produce secondary products, such as shikonin, diosgenin, caffeine, glutathione, capsaicin, and anthraquinone [158–160]. Large-scale production of such compounds (molecular farming) is increasingly becoming popular with the industry where some physical and chemical conditions for growth and product formation have been optimized. Hairy root cultures, which are considered genetically more stable, are now increasingly being used for production of secondary metabolites *in vitro* [161].

16.11 Cryopreservation and *In Vitro* Germplasm Storage

The aim of germplasm conservation is to ensure the ready availability of useful germplasm for scientific research. In seed-propagated crops, seed is extensively used for conservation of germplasm through conventional methods. However, in vegetatively propagated species, where conventional storage techniques are used, it is very difficult to store germplasm on a long-term basis. The conservation of plant parts *in vitro* has a number of advantages over *in vivo* conservation; for example, *in vitro* techniques allow conservation of plant species that are in danger of becoming extinct. *In vitro* storage of vegetatively propagated plants can result in great savings in storage space and time and sterile plants that cannot be reproduced generatively can be maintained *in vitro*. Complete plants have been successfully regenerated from tissues cryopreserved at −196 °C in liquid nitrogen (LN) for several months to years in several crops [162, 163]. This method is now being practically used at several national and international germplasm banks. Successful cryopreservation of plant shoot tips depends upon effective desiccation through osmotic or physical processes. Cryoprotective treatments, which favor survival of small, meristematic, and young leaf cells, are most likely to produce high survival rates after exposure to liquid nitrogen. Furthermore, microscopy techniques have been used to determine the extent of cellular damage and plasmolysis that occur in peppermint (*Mentha piperita*) shoot tips during the process of cryopreservation, using cryoprotectant plant vitrification solution 2 (PVS2) (30% glycerol, 15% dimethyl sulfoxide, 15% ethylene glycol, and 0.4 M sucrose) prior to liquid nitrogen exposure [164]. *Arabidopsis*, which is increasingly being used in genomic studies, can be successfully cryopreserved using either PVS2 or PVS3 as cryoprotectants prior to rapidly cooling shoot tips in LN. PVS3 contains 50% glycerol compared to PVS2 that contains 30% glycerol. PVS3 is less injurious than PVS2. All the shoot tips regrew after LN exposure when cryoprotected with PVS3 for 60 min at 22 °C [165]. The high levels of shoot formation after LN exposure of *Arabidopsis* shoot tips make this a desirable system, in which molecular tools can be used to examine how alterations in biochemical, metabolic, and development processes affect regrowth after cryoprotective treatments. The method has been used for cryopreservation of rice [166], potato [167–169], citrus [170], and blackberry and raspberry [171].

Table 16.3 Tissue culture-based vector and vectorless gene transfer methods in plants.

Vector methods	Vectorless methods
1. *Agrobacterium tumefaciens*	1. Physicochemical uptake of DNA
2. *A. rhizogenes*	2. Liposome encapsulation
3. Sonication-assisted *Agrobacterium* method	3. Electroporation of protoplasts
4. Viral vectors	4. Microinjection
	5. DNA injection into intact plants
	6. Incubation of seeds with DNA
	7. Pollen tube pathway
	8. Use of laser microbeam
	9. Electroporation into tissues/embryos
	10. Silicon carbide fiber method
	11. Particle bombardment

16.12 Genetic Transformation

During the past 20 years, the combined use of recombinant DNA technology, gene transfer methods (Table 16.3), and tissue culture techniques has led to the efficient transformation and production of transgenics in a wide variety of crop plants. In fact, transgenesis has emerged as an additional tool to carry out single-gene breeding or transgenic breeding of crops. Unlike conventional breeding, only the cloned genes of agronomic importance are being introduced without cotransfer of undesirable genes from the donor. The recipient genotype is least disturbed, which eliminates the need for repeated backcrosses. Above all, the transformation method provides access to a large gene pool, as the gene(s) may come from viruses, bacteria, fungi, insects, animals, human beings, unrelated plants, and even from chemical synthesis in the laboratory. Various gene transfer methods [172–175] have been developed for genetic transformation of plants. Among these, *Agrobacterium* and "particle gun" (Figure 16.10) methods are being widely used.

16.12.1 Significance of Plant Genetic Transformation

Rapid and remarkable achievements have been made in the production, characterization, and field evaluation of transgenic plants in several field and fruit crops and forest plant species. Using different gene transfer methods and strategies, transgenics carrying useful agronomic traits have been developed and released in several crops. Transgenic varieties, possessing mainly insect resistance, herbicide resistance, or both, disease resistance, of 10 crops are now being commercially grown in an area of about 134 million ha across 25 countries [176]. Attempts are being made not only to develop transgenic crop varieties resistant to abiotic stresses, such as drought, low and high temperature, salts, and heavy metals but also to develop

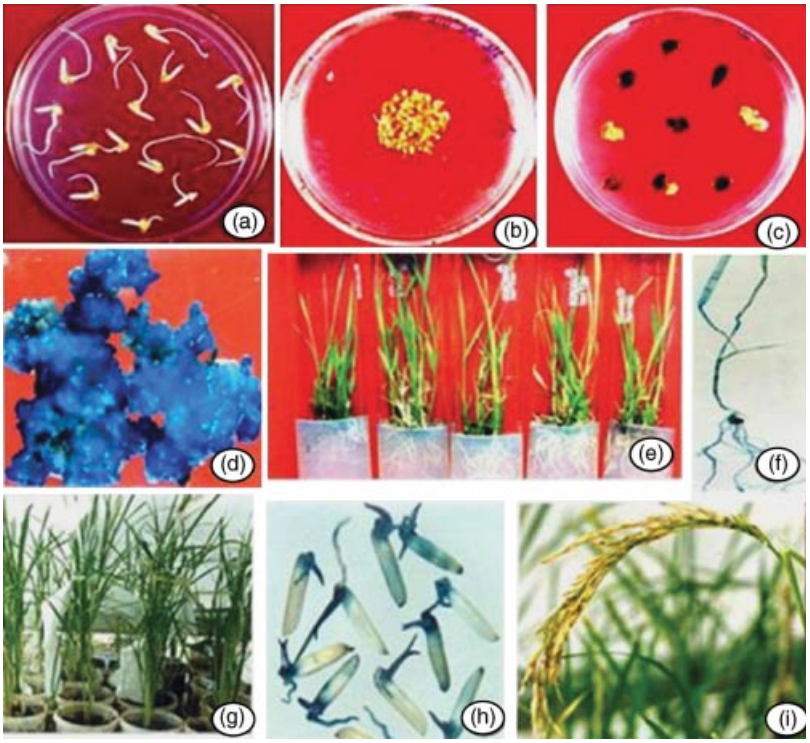

Figure 16.10 Genetic transformation of rice. (a) Germinating mature grains cultured *in vitro*. (b) Scutellar-derived calli placed in the center of target plate. (c) Bombarded calli cultured in selection medium showing blackening of nontransformed calli. (d) GUS expression in the selected callus. (e) Plant regeneration from selected calli. (f) Regenerated plantlet showing GUS exporession. (g) Putative transgenic T_0 plants in the green house. (h) Germinating T_1 grains showing GUS expression. (i) Transgenic plant showing fully fertile mature spike.

transgenic varieties possessing better nutrient use efficiency and better keeping, nutritional, and processing qualities. Genetically modified foods, such as tomato containing high lycopene, flavonols as antioxidants, cavity-fighting apples, golden rice with enhanced vitamin A, golden brassica with provitamin A, canola rich in vitamin E, proteinaceous potatoes, edible vaccines, decaffeinated tea and coffee, and nicotine-free tobacco, are leading examples of genetically engineered crops.

References

1 Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.*, **15**, 473–497.

2 White, P.R. (1963) *The Cultivation of Animal and Plant Cells*, Ronald Press, New York.

3 Gamborg, O.L., Miller, R.A., and Ojima, K. (1968) Nutrient requirements

of suspension cultures of soyabean root cells. *Exp. Cell Res.*, **50**, 151–158.
4 Chu, C.C. (1978) The N6 Medium and Its Applications to Anther Culture of Cereal Crops, in Proc. Symp. Plant Tissue Culture Science Press, Beijing, pp. 45–50.
5 Chahal, G.S. and Gosal, S.S. (2002) *Principles and Procedures of Plant Breeding: Biotechnological and Conventional Approaches*, Narosa Publ. House, New Delhi.
6 Kang, M.S., Subudhi, P.K., Baisakh, N., and Priyadarshan, P.M. (2007) Crop breeding methodologies: classic and modern, in *Breeding Major Food Staples* (eds M.S. Kang and P.M. Priyadarshan), Blackwell, USA, pp. 5–40.
7 Gosal, S.S., Thind, K.S., and Dhaliwal, H.S. (1998) Micropropagation of sugarcane: an efficient protocol for commercial plant production. *Crop Improv.*, **25**, 1–5.
8 Gill, R., Malhotra, P.K., and Gosal, S.S. (2006b) Direct plant regeneration from cultured young leaf segments of sugarcane. *Plant Cell Tissue Organ Cult.*, **84**, 227–231.
9 Sood, N., Piyush, K., Srivastava, R.K., and Gosal, S.S. (2006) Comparative studies on field performance of micropropagated and conventionally propagated sugarcane plants. *Plant Tissue Cul. Biotechnol.*, **16**, 25–29.
10 Jalaja, N.C., Sreenivasan, T.V., Pawar, S.M., Bhoi, P.G., and Garker, R.M. (2006) Co 94012: a new sugarcane variety through somaclonal variation. *Sugar Tech.*, **8**, 132–136.
11 Jalaja, N.C., Neelamathi, D., and Sreenivasan, T.V. (2008) Micropropagation for quality seed production in sugarcane in Asia and the pacific. Food and Agriculture Organization of the United Nations, Rome; Asia-Pacific Consortium on Agricultural Biotechnology, New Delhi; Asia- Pacific Association of Agricultural Research Institutions. i–x, p. 46. Bangkok.
12 Cronauer, S.S. and Krikorian, A.D. (1984) Multiplication of Musa from excised stem tips. *Ann. Bot.*, **53**, 321–328.
13 Damasco, O.P., Smith, M.K., Godwin, I.D., Adkins, S.W., Smillie, R.M., and Hetherington, S.E. (1997) Micropropagated dwarf off-type Cavendish bananas (*Musa* spp., AAA) show improved tolerance to suboptimal temperatures. *Aust. J. Agric. Res.*, **48**, 377–384.
14 Kodym, A. and Zapata-Arias, F.J. (1999) Natural light as an alternative light source for the *in vitro* culture of banana (*Musa acuminata* cv. "Grande Naine"). *Plant Cell Tissue Organ Cult.*, **55**, 141–145.
15 Kalimuthu, K., Saravanakumar, M., and Senthikumar, R. (2007) *In vitro* micropropagation of *Musa sapientum* L. (Cavendish Dwarf). *Afr. J. Biotech.*, **6**, 1106–1109.
16 Resmi, L. and Nair, A.S. (2007) Plantlet production from male inflorescence tips of *Musa acuminata* cultivars from South India. *Plant Cell Tissue Organ Cult.*, **88**, 333–338.
17 Farahani, F., Aminpoor, H., Sheidai, M., Noormohammadi, Z., and Mazinani, M.H. (2008) An improved system for *in vitro* propagation of banana (*Musa acuminata* L.) cultivars. *Asian J. Plant Sci.*, **7**, 116–118.
18 Pereira, J.E.S. and Fortes, G.R. (2004) Production of pre-basic potato plants by cuttings obtained from micropropagated plants. *Horti. Brasil.*, **22**, 186–192.
19 Ebadi, M., Iranbakhsh, A., and Khaniki, G.B. (2007) Shoot micropropagation and microtuberization in potato (*Solanum tuberosum* L.) by the semi-continuous bioreactor. *Pak. J. Biol. Sci.*, **10**, 861–867.
20 Badoni, A. and Chauhan, J.S. (2009) Single node callus culture: improvement for micropropagation of *Solanum tuberosum* (cv. *Kufri Himalini*). *Nat. Sci.*, **7**, 99–103.
21 Chopra, H.R., Dhaliwal, H.S., and Gosal, S.S. (1993) Micropropagation: an efficient method to multiply strawberries. *Punjab Veget. Grower*, **28**, 9–11.
22 Mohamed, A.E. (2007) Somaclonal variation in micro-propagated strawberry detected at the molecular level. *Int. J. Agric. Biol.*, **9**, 721–725.

23 Gill, R.I.S., Gill, S.S., and Gosal, S.S. (1994) Vegetative propagation of *Eucalyptus tereticornis* Sm. through tissue culture. *Plant Tissue Cult.*, **4**, 59–67.

24 Gill, S.S., Gill, R.I.S., and Gosal, S.S. (1997) Rapid propagation of *Dalbergia sissoo* from mature trees through tissue culture. *Plant Tissue Cult.*, **7**, 13–19.

25 Sooch, M., Arora, J.S., Singh, K., and Gosal, S.S. (1998) Studies on *in vitro* propagation of carnation (*Dianthus caryophyllus* L.). *Plant Tissue Cult.*, **8**, 61–68.

26 Chaturvedi, H.C., Sharma, A.K., Agha, B.Q., Jain, M., and Sharma, M. (2004) Production of cloned trees of *Populus deltoides* through *in vitro* regeneration of shoots from leaf, stem and root explants and their field cultivation. *Ind. J. Biotech.*, **3**, 203–208.

27 Gill, R., Gupta, P.K., and Gosal, S.S. (2004b) Brahmi: efficient protocol for micropropagation. *Indian Med. Aromatic Plants Today*, 3–5.

28 Gosal, S.S. and Gill, R. (2004) Different biotechnological options for improving medicinal and aromatic plants. *Indian Med. Aromatic Plants Today*, 19–21.

29 Gill, M.K., Chauhan, S.K., and Gosal, S.S. (2006a) Macro- and micro-propagation of *Azadirachta indica*. *Indian Forester*, **132**, 1159–1168.

30 Machado, M.P., Biasi, L.A., Ritter, M., Ribas, L.L.F., Koehler, H.S., and Zanette, F. (2007) Culture media in micropropagation of grapevine rootstock "VR043-43" (*Vitis vinifera* × *Vitis rotundifolia*). *Ciencia Rural*, **37**, 277–280.

31 Jaskani, M.J., Abbas, H., Sultana, R., Khan, M.M., Qasim, M., and Khan, I.A. (2008) Effect of growth hormones on micropropagation of *Vitis vinifera* L. cv. Perlette. *Pak. J. Bot.*, **40**, 105–109.

32 Thind, S.K., Jain, N., and Gosal, S.S. (2007) *Aloe vera* L. clones produced *in vitro* and under natural conditions vary in carbohydrate accumulation. *J. Plant Sci. Res.*, **23**, 41–45.

33 Liu, Z. and Gao, S. (2007) Micropropagation and induction of autotetraploid plants of *Chrysanthemum cinerariifolium* (Trev.) Vis. *In Vitro Cell. Dev. Biol. Plant*, **43**, 404–408.

34 Sandhu, J.S., Gosal, S.S., Gill, M.S., and Dhaliwal, H.S. (1995) Micropropagation of *Indica* rice through proliferation of axillary shoots. *Euphytica*, **81**, 139–142.

35 Medina, R., Faloci, M., Marassi, M.A., and Mroginski, L.A. (2004) Genetic stability in rice micropropagation. *Biocell*, **28**, 13–20.

36 Murashige, T. (1980) Plant growth substances in commercial uses of tissue culture, in *Plant Growth Substances* (ed. F. Skoog), Springer, Berlin, pp. 426–434.

37 Schenck, S. and Lehrer, A. (2000) Factors affecting the transmission and spread of sugarcane yellow leaf virus. *Plant Dis.*, **84**, 1085–1088.

38 Morel, G. and Martin, C. (1952) Guerison de dahlias atteints d'une maladie a virus. *C.R. Hebd. Séances Acad. Sci. (Paris)*, **234**, 1324–1325.

39 Mori, K. and Hosokawa, D. (1977) Localization of viruses in apical meristem and production of virus-free plants by means of meristem and tissue culture. *Acta Hort.*, **78**, 389–396.

40 Fitch, M.M.M., Lehrer, A.T., Komor, E., and Moore, P.H. (2001) Elimination of sugarcane yellow leaf virus from infected sugarcane plants by meristem tip culture visualized by tissue blot immunoassay. *Plant Pathol.*, **50**, 676–680.

41 Balamuralikrishnan, M., Doraisamy, S., Ganapathy, T., and Viswanathan, R. (2002) Combined effect of chemotherapy and meristem culture on sugarcane mosaic virus elimination in sugarcane. *Sugar Tech.*, **4**, 19–25.

42 Gupta, P.P. (1986) Eradication of mosaic disease and rapid clonal multiplication of bananas and plantains through meristem tip culture. *Plant Cell Tissue Organ Cult.*, **6**, 33–39.

43 Allam, E.K., Othman, B.A., Sawy, E.I., and Thabet, S.D. (2000) Eradication of banana bunchy top virus (BBTV) and banana mosaic virus (BMV) from diseased banana plants. *Ann. Agril. Sci. Cairo*, **45**, 33–48.

44 Faccioli, G. and Colombarini, A. (1996) Correlation of potato virus S and virus M contents of potato meristem tips with the percentage of virus-free plantlets

produced *in vitro*. *Potato Res.*, **39**, 129–140.
45 Thind, S.K., Ghai, M., and Gosal, S.S. (2005) Factors affecting *in vitro* growth of meristem tip-derived plantlets of *Solanum tuberosum*. cvs. *Phytomorphology*, **55**, 211–220.
46 Theiler-Hedtrich, R. and Baumann, G. (2008) Elimination of apple mosaic virus and raspberry bushy dwarf virus from infected red raspberry (*Rubus idaeus* L.) by tissue culture. *J. Phytopathol.*, **127**, 193–199.
47 Williams, E.G. and Maheswaran, G. (1986) Somatic embryogenesis: factors influencing co-ordinated behaviour of cells as an embryogenic group. *Ann. Bot.*, **57**, 443–462.
48 Abdullah, R., Cocking, E.C., and Thompson, J.A. (1986) Efficient plant regeneration from rice protoplasts through somatic embryogenesis. *Biotechnology*, **4**, 1087–1090.
49 Grewal, D.K., Gill, R., and Gosal, S.S. (2006a) Influence of antibiotic cefotaxime on somatic embryogenesis and plant regeneration in Indica rice. *Biotechnol. J.*, **1**, 1158–1162.
50 Greer, M.S., Igor, K., and Eudes, F. (2009) Ammonium nitrate improves direct somatic embryogenesis and biolistic transformation of *Triticum aestivum*. *New Biotechnol.*, **26**, 44–52.
51 Gill, N.K., Gill, R., and Gosal, S.S. (2004a) Factors enhancing somatic embryogenesis and plant regeneration in sugarcane (*Saccharum officinarum* L.). *Indian J. Biotechnol.*, **3**, 119–123.
52 Kaur, A. and Gosal, S.S. (2009) Desiccation of callus enhances somatic embryogenesis and subsequent shoot regeneration in sugarcane. *Indian J. Biotechnol.*, **8**, 332–334.
53 Hodges, T.K., Kamo, K.K., Imbrie, C.W., and Becwar, M.R. (1986) Genotype specificity of somatic embryogenesis and regeneration in maize. *Biotechnol.*, **4**, 219–223.
54 Deng, S., Dong, Z., Zhan, K., Hu, Y., Yin, D., and Cui, D. (2009) Moderate desiccation dramatically improves shoot regeneration from maize (*Zea mays L.*) callus. *In Vitro Cell. Dev. Plant*, **45**, 99–103.
55 Zhang, B.-H., Liu, F., and Yao, C.-B. (2000) Plant regeneration via somatic embryogenesis in cotton. *Plant Cell Tissue Organ Cult.*, **60**, 89–94.
56 Zhang, B., Wang, Q., Liu, F., Wang, K., and Frazier, T.P. (2009) Highly efficient plant regeneration through somatic embryogenesis in 20 elite commercial cotton (*Gossypium hirsutum* L.) cultivars. *Plant Omics J.*, **2**, 259–268.
57 Gill, M.I.S., Singh, Z., Dhillon, B.S., and Gosal, S.S. (1995) Somatic embryogenesis and plantlet regeneration in mandarin (*Citrus reticulata* Blanco). *Sci. Hortic.*, **63**, 167–174.
58 Gosal, S.S., Gill, M.I.S., and Grewal, H.S. (1995) Somatic embryogenesis in citrus species, in *Somatic Embryogenesis in Woody Plants, Angiosperms*, vol. 2 (eds S. Jain, P. Gupta, and R. Newton), Kluwer Academic Publishers, The Netherlands, pp. 1–21.
59 Larkin, P.J. and Scowcroft, W.R. (1981) Somaclonal variation: a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.*, **60**, 197–214.
60 Lee, M. and Phillips, R.L. (1988) The chromosomal basis of somaclonal variation. *Ann. Rev. Plant Physiol. Plant Mol. Biology*, **39**, 413–437.
61 Evans, D.A., Sharp, W.R., and Medina Filho, H.P. (1984) Somaclonal and gametoclonal variation. *Am. J. Bot.*, **6**, 759–774.
62 Dulieu, H. and Barbier, M. (1982) High frequencies of genetic variant plants regenerated from cotyledons of tobacco, in *Variability in Plants Regenerated from Tissue Culture* (eds E.D. Earley and Y. Demarley), Praeger Press, New York, pp. 211–299.
63 Evans, D.A. and Sharp, W.R. (1986) Somaclonal variation in agriculture. *Biotechnology*, **4**, 528–532.
64 Gengenbach, B.G., Green, C.E., and Donovan, C.M. (1977) Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. *Proc. Natl. Acad. Sci. USA*, **74**, 5113–5117.
65 Santoso, D. and Thornburg, R. (2002) Fluoroorotic acid-selected *Nicotiana plumbaginifolia* cell lines with a stable

thymine starvation phenotype have lost the thymine-regulated transcriptional program. *Plant Physiol.*, **123** (4), 1517–1524.

66 Kaeppler, S.M., Kaeppler, H.F., and Rhee, Y. (2000) Epigenetic aspects of somaclonal variation in plants. *Plant Mol. Biol.*, **43**, 179–188.

67 Bardini, M., Labra, M., Winfield, M., and Sala, F. (2003) Antibiotic-induced DNA methylation changes in calluses of *Arabidopsis thaliana*. *Plant Cell Tissue Organ Cult.*, **72**, 157–162.

68 Bednarek, P.T., Orłowska, R., Koebner, R.M., and Zimny, J. (2007) Quantification of the tissue-culture induced variation in barley (*Hordeum vulgare* L.). *BMC Plant Biol.*, **7**, 10–16.

69 Larkin, P.J., Banks, P.M., Bhati, R., Brettell, R.I.S., Davies, P.A., Ryan, S.A., Scowcroft, W.R., Spindler, L.H., and Tanner, G.J. (1989) From somatic variation to variant plants: mechanisms and applications. *Genome*, **31**, 705–711.

70 Jain, S.M., Brar, D.S., and Ahloowalia, B.S. (1989) *Somaclonal Variation and Induced Mutations in Crop Improvement*, Kluwer Academic Publishers, The Netherlands.

71 Leal, M.R., Maribona, R.H., Ruiz, A., Korneva, S., Canales, E., Dinkova, T.D., Izquierdo, F., Goto, O., and Rizo, D. (1994) Somaclonal variation as a source of resistance to eyespot disease of sugarcane. *Plant Breed.*, **115**, 37–42.

72 Kaur, A., Gosal, S.S., Gill, R., and Thind, K.S. (2001) Induction of plant regeneration and somaclonal variation for some agronomic traits in sugarcane (*Saccharum officinarum* L.). *Crop Improv.*, **28**, 167–172.

73 Khan, S.J., Khan, M.A., Ahmad, H.K., Khan, R.D., and Zafar, Y. (2004) Somaclonal variation in sugarcane through tissue culture and subsequent screening for salt tolerance. *Asian J. Plant Sci.*, **3**, 330–334.

74 Doule, R.B. (2006) Cane yield and quality characters of some promising somaclonal variants of sugarcane. *Sugar Tech.*, **8**, 191–193.

75 Singh, G., Sandhu, S.K., Meeta, M., Singh, K., Gill, R., and Gosal, S.S. (2008) *In vitro* induction and characterization of somaclonal variation for red rot and other agronomic traits in sugarcane. *Euphytica*, **160**, 35–47.

76 Sengar, A.S., Thind, K.S., Kumar, B., Mittal, P., and Gosal, S.S. (2009) *In vitro* selection at cellular level for red rot resistance in sugarcane (*Saccharum* sp.). *Plant Growth Regul.*, **58**, 2001–2009.

77 Das, A., Gosal, S.S., Sidhu, J.S., and Dhaliwal, H.S. (2000) Induction of mutations for heat tolerance in potato by using *in vitro* culture and radiation. *Euphytica*, **114**, 205–209.

78 Veitia-Rodriguez, N., Francisco-Cardoso, J., Perez, J.N., Garcia-Rodriguez, L., Bermudez-Caraballosos, I., Garcia-Rodriguez, L., Padron-Montesinos, Y., Orellana-Perez, P., Romero-Quintana, C., and Hernandez, N. (2002) Evaluations in field of somaclones of Irish potatoes (*Solanum tuberosum* Lin.) of the variety Desiree obtained by somaclonal variation and *in vitro* mutagenesis. *Biotecnol. Veget.*, **2**, 21–26.

79 Lara, R.M., Lorido, M., Plana, D., More, O., Gonzalez, M.E., Alvarez, M., and Hernandez, M.M. (2003) Isoenzymatic analysis for detecting *in vitro* variability and/or stability of economically important crops. *Cultivos Tropic.*, **24**, 39–47.

80 Zheng, X., Wei-Xiao, M., Ji-Liang, Y., and Hu-Yan, M. (2004) *In vitro* selection of NaCl-tolerant variants of maize and analysis of salt tolerance. *J. Henan Agri. Univ.*, **38**, 139–143.

81 Araujo, L.G. and Prabhu, A.S. (2004) Partial resistance to blast in somaclones of rice cultivar CICA-8. *Fitopatol. Bras.*, **29**, 394–398.

82 Cristo, E., Gonzalez, M.C., and Perez, A.V. (2006) Obtaining somaclones derived from rice (*Oryza sativa* L.) plants through anther culture of hybrids and varieties. *Cultivos Tropic.*, **27**, 35–39.

83 Elanchezhian, R. and Mandal, A.B. (2007) Growth analysis of somaclones regenerated from a salt tolerant traditional "Pokkali" rice (*Oryza sativa*). *Indian J. Agric. Sci.*, **77**, 184–187.

84 Ahmed, K.Z. and Abdelkareem, A.A. (2005) Somaclonal variation in bread wheat (*Triticum aestivum* L.). II. Field performance of somaclones. *Cereal Res. Commun.*, **33**, 485–492.

85 Sabry, S.R.S., Moussa, A.M., Menshawy, A.M., and El-Borhami, H.S. (2005) Regeneration of leaf rust (*Puccinia recondita*) resistant high-yielding wheat (*Triticum aestivum* L.) somaclones from embryogenic callus of Sakha 61 cultivar. *Bull. Faculty Agri. Cairo Univ.*, **56** (3), 469–481.

86 Rozhanskaya, O.A. (2006) Quantitative variability in populations of alfalfa somaclones and mutants. *Russian Agric. Sci.*, **2**, 4–7.

87 Hammerschlag, F.A. and Ognjanov, V. (1990) Somaclonal variation in peach: screening for resistance to *Xanthomonas campestris* pv. *pruni* and *Pseudomonas syringae* pv. *syringae*. *Acta Hort.*, **280**, 403–408.

88 Rosati, P. and Predieri, S. (1990) *In vitro* selection of apple rootstock somaclones with *Phytophthora cactorum* culture filtrate. *Acta Hort.*, **280**, 409–413.

89 Donovan, A.M., Morgan, R., Piagnani, V., Ridout, M.S., James, D.J., and Garrett, C.M.E. (1994) Assessment of somaclonal variation in apple. I. Resistance to fire blight pathogen *Erwinia amylora*. *J. Hort. Sci.*, **69**, 105–113.

90 Larkin, P.J., Li, Y., Spindler, L.H., Tanner, G.J., and Banks, P.M. (1993) Disease resistance, cell culture and somatic recombination. *Acta Hort.*, **336**, 341–346.

91 Karp, A. (1995) Somaclonal variation as a tool for crop improvement. *Euphytica*, **85**, 295–302.

92 Khush, G.S. and Virk, P.S. (2002) Rice improvement: past, present and future, in *Crop Improvement Challenges in the Twenty-First Century* (ed. S. Kang Manjit), The Haworth Press, Inc., New York, pp. 17–42.

93 Gosal, S.S., Sindhu, A.S., Sandhu, J.S., Gill, R., Khehra, G.S., Sidhu, G.S., and Dhaliwal, H.S. (1996) Haploidy in rice, in *In Vitro Haploid Production in Higher Plants* (eds S.M. Jain, S.K. Sopory, and R.E. Veilleux), Kluwer Academic Publishers, The Netherlands, pp. 1–37.

94 Gill, R., Kaur, N., Sindhu, A.S., Bharaj, T.S., and Gosal, S.S. (2003) in *Advances in Rice Genetics* (eds G.S. Khush, D.S. Brar, and B. Hardy), International Rice Research Institute, Manila, Philippines.

95 Guha, S. and Maheshwari, S.C. (1964) *In vitro* production of embryos from anthers of *Datura*. *Nature*, **204**, 497.

96 Guha, S. and Maheshwari, S.C. (1966) Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. *Nature*, **212**, 97–98.

97 De Buyser, J., Henry, Y., Lonnet, P., Hertzog, P., and Hespel, A. (1986) "Florin": a doubled haploid wheat variety developed by the anther culture method. *Plant Breed.*, **98**, 53–56.

98 Laurie, D.A. and Reymondie, S. (1991) High frequencies of fertilization and haploid seedling production in crosses between commercial hexaploid wheat varieties and maize. *Plant Breed.*, **106**, 182–189.

99 García-llamas, C., Martín, A., and Ballesteros, J. (2004) Differences among auxin treatments on haploid production in durum wheat × maize crosses. *Plant Cell Rep.*, **23**, 46–49.

100 Mujeeb-Kazi, A., Gul, A., Ahmed, J., and Mirza, J.I. (2006) A simplified and effective protocol for production of bread wheat haploids ($n = 3x = 21$, ABD) with some application areas in wheat improvement. *Pak. J. Bot.*, **38**, 393–406.

101 Obert, B. and Barnabas, B. (2004) Colchicine induced embryogenesis in maize. *Plant Cell Tissue Organ Cult.*, **77**, 283–285.

102 Ambrus, H., Darko, E., Szabo, L., Bakos, F., Kiraly, Z., and Barnabas, B. (2006) *In vitro* microspore selection in maize anther culture with oxidative-stress stimulators. *Protoplasma*, **228**, 87–94.

103 Bernardo, R. (2009) Should maize doubled haploids be induced among F_1 or F_2 plants? *Theor. Appl. Genet.*, **119**, 255–262.

104 Raina, S.K. and Zapata, F.J. (1997) Enhanced anther culture efficiency of *indica* rice (*Oryza sativa* L.) through modification of the culture media. *Plant Breed.*, **116**, 305–315.

105 Senadhira, D., Zapata-Arias, F.J., Gregoric, G.B., Alejar, M.S., de la Cruz, H.C., Padolina, T.F., and Galvez, A.M. (2002) Development of the first salt-tolerant rice cultivar through *indica*/*indica* anther culture. *Field Crop Res.*, **76**, 103–110.

106 Sarao, N.K., Gill, M.S., Gill, R., Bharaj, T.S., and Gosal, S.S. (2003) An improved method for pollen culture in rice. *Oryza*, **40**, 77–79.

107 Grewal, D.K., Gill, R., and Gosal, S.S. (2006) Role of cysteine in enhancing androgenesis and regeneration of *indica* rice (*Oryza sativa* L.). *Plant Growth Regul.*, **49**, 43–47.

108 Zhahg-Yi, Y., Hong-Ru, K., Zhahg-Jin, W., Li-Zheng, Y., and Zeng-Qian, C. (2008) High quality and blast resistance DH lines via anther culture. *Southwest China J. Agrl. Sci.*, **21**, 75–79.

109 Seguı-Simarro, J.M. and Nuez, F. (2007) Embryogenesis induction, caulogenesis, and plant regeneration by *in vitro* culture of tomato isolated microspores and whole anthers. *J. Expt. Bot.*, **58**, 1119–1132.

110 Germanà, M.A., Chiancone, B., Lain, O., and Testolin, R. (2005) Anther culture in *Citrus clementina*: a way to regenerate tri-haploids. *Aust. J. Agric. Res.*, **56**, 839–845.

111 Antonietta, G.M., Benedetta, C., Calogero, I., and Rosario, M. (2005) The effect of light quality on anther culture of *Citrus clementina* Hort. ex Tan. *Acta Physiol. Plant.*, **27**, 717–721.

112 Jain, S.M., Sopory, S.K., and Veilleux, R.E. (1996) *In Vitro Haploid Production in Higher Plants*, Kluwer Academic Publishers, The Netherlands.

113 Guzmán, M. and Zapata-Arias, F.J. (2000) Increasing anther culture efficiency in rice (*Oryza sativa* L.) using anthers from ratooned plants. *Plant Sci.*, **151**, 107–114.

114 Nitsch, J.P. and Nitsch, C. (1969) Haploid plants from pollen grains. *Science*, **163**, 85–87.

115 Cho, M.S. and Zapata, F.J. (1990) Plant regeneration from isolated microspores of *Indica* rice. *Plant Cell Physiol.*, **31**, 881–885.

116 Huang, B., Bird, S., Kemble, R., Simmonds, D., Keller, W., and Miki, B. (1990) Effects of culture density, conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus* L. cv. Topas. *Plant Cell Rep.*, **8**, 594–597.

117 Binarova, P., Straatman, K., Hause, B., Hause, G., and Lammeren, A.A.M. (1993) Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L. *Theor. Appl. Genet.*, **87**, 9–16.

118 Chen, Z.Z., Snyder, Z., Fan, Z.G., and Loh, W.H. (1994) Efficient production of doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. *Plant Breed.*, **113**, 217–221.

119 Malik, M.R., Wang, F., Dirpaul, J.M., Zhou, N., Polowick, P.L., Ferrie, A.M.R., and Krochko, J.E. (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. *Plant Physiol.*, **144**, 134–154.

120 Agarwal, P.K. and Bhojwani, S.S. (1993) Enhanced pollen grain embryogenesis and plant regeneration in anther cultures of *Brassica juncea* cv. PR-45. *Euphytica*, **70**, 191–196.

121 Agarwal, P.K. and Bhojwani, S.S. (2004) Genetic variability in the progeny of androgenic dihaploid plants and selection of high agronomic performing lines in *Brassica juncea*. *Biol. Plant.*, **48**, 503–508.

122 Chanana, N.P., Dhawan, V., and Bhojwani, S.S. (2005) Morphogenesis in isolated microspore cultures of *Brassica juncea*. *Plant Cell Tissue Organ Cult.*, **83**, 169–177.

123 Agarwal, P.K., Agarwal, P., Custers, J.B.M., Liu, C.M., and Bhojwani, S.S. (2006) PCIB an antiauxin enhances microspore embryogenisis in microspore culture of *Brassica juncea*. *Plant Cell Tissue Organ Cult.*, **86**, 201–210.

124 Hoekstra, S., van Zijderveld, M.H., Heidekamp, E., and van der Mark, E. (1993) Microspore culture of *Hordeum vulgare* L.: the influence of density and osmolality. *Plant Cell Rep.*, **12**, 661–665.

125 Hu, T. and Kasha, K.J. (1997) Improvement of isolated microspore culture of wheat (*Triticum aestivum* L.)

through ovary co-culture. *Plant Cell Rep.*, **16**, 520–525.

126 Sidhu, P.K. and Davies, P.A. (2009) Regeneration of fertile green plants from oat isolated microspore culture. *Plant Cell Rep.*, **28**, 571–577.

127 Lantos, C., Juhász, A.G., Ötvös, G.S.K., Vági, P., Mihály, R., Kristóf, Z., Somogyi, N., and Pauk, J. (2009) Improvement of isolated microspore culture of pepper (*Capsicum annuum* L.) via co-culture with ovary tissues of pepper or wheat. *Plant Cell Tissue Organ Cult.*, **97**, 285–293.

128 Tang, F., Tao, Y., Zhao, T., and Wang, G. (2006) *AT In vitro* production of haploid and doubled haploid plants from pollinated ovaries of maize (*Zea mays*). *Plant Cell Tissue Organ Cult.*, **84**, 233–237.

129 Zhou, C. and Yang, H.Y. (1981) Embryogenesis in unfertilized embryo seed of rice. *Acta Bot. Sin.*, **23**, 176–180.

130 Kasha, K.J. and Kao, K.N.M. (1970) High frequency of haploid production in barley (*Hordeum vulgare* L.). *Nature*, **225**, 874–875.

131 Barclay, I.R. (1975) High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination. *Nature*, **256**, 410–411.

132 Zenkteler, M. and Nitzsche, W. (1984) Wide hybridization experiments in cereals. *Theor. Appl. Genet.*, **68**, 311–315.

133 Zenkteler, M. and Nitzsche, W. (1985) *In vitro* culture of ovules of *Triticum aestivum* at early stages of embryogenesis. *Plant Cell Rep.*, **4**, 168–171.

134 Laurie, D.A. and Bennet, M.D. (1986a) Wheat × maize hybridization. *Can. J. Genet. Cytol.*, **28**, 313–316.

135 Laurie, D.A. and Bennett, M.D. (1986b) The production of haploid wheat plants from wheat × maize crosses. *Theor. Appl. Genet.*, **76**, 393–397.

136 O'Donoughue, L.S. and Bennett, M.D. (1994) Durum wheat haploid production using maize wide-crossing. *Theor. Appl. Genet.*, **89**, 559–566.

137 Bains, N.S., Singh, J., Ravi, and Gosal, S.S. (1995) Production of wheat haploids through embryo rescue from wheat × maize crosses. *Curr. Sci.*, **69**, 621–623.

138 Verma, V., Bains, N.S., Mangat, G.S., Nanda, G.S., Gosal, S.S., and Singh, K. (1999) Maize genotypes show striking differences for induction and regeneration of haploid wheat embryos in the wheat × maize system. *Crop Sci.*, **39**, 1722–1727.

139 Sharma, D.R., Kaur, R., and Kumar, K. (1996) Embryo rescue in plants. *Euphytica*, **89**, 325–337.

140 Takahata, Y. and Takeda, T. (1990) Intergeneric (intersubtribe) hybridization between *Moricandia arvensis* and *Brassica* A and B genome species by ovary culture. *Theor. Appl. Genet.*, **80**, 38–42.

141 Momotaz, A., Kato, M., and Kakihara, F. (1998) Production of intergeneric hybrids between *Brassica* and *Sinapis* species by means of embryo rescue techniques. *Euphytica*, **103**, 123–130.

142 Kaur, J., Satija, C.K., and Gosal, S.S. (2002) *In vitro* synthesis of white grained primary hexaploid triticales. *Plant Tissue Cult.*, **12**, 1–9.

143 Gosal, S.S. and Bajaj, Y.P.S. (1983) Interspecific hybridization between *Vigna mungo* and *Vigna radiata* through embryo culture. *Euphytica*, **32**, 129–137.

144 Moss, J.P., Stalker, H.T., and Pattee, H.E. (1988) Embryo rescue in wide crosses in *Arachis*. 1. Culture of ovules in peg tips of *Arachis hypogaea*. *Ann. Bot.*, **61**, 1–7.

145 Van Tuyl, J.M., Van Dien, M.P., Van Creij, M.G.M., Van Kleinwee, T.C.M., Franken, J., and Bino, R.J. (1991) Application of *in vitro* pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lillium* crosses. *Plant Sci.*, **74**, 115–126.

146 Calagari, M., Jafari Mofidabadi, A., Tabari, M.., and Hosseini, S.M. (2004) Intraspecific hybridization of *Populus euphratica* Oliv. using *in vitro* technique. *J. Sci. Islamic Repub. Iran*, **15**, 109–112.

147 Singh, Z.S., Brar, J.S., and Gosal, S.S. (1991) Ovule culture of seedless grapes (*Vitis vinifera* L.) cv. Perlette. *Acta Hortic.*, **300**, 325–329.

148 Hinnisdaels, S., Negrutiv, I., Jacobs, M., and Sidorov, V. (1988) Plant somatic cell

hybridizations: evaluations and perspectives. *IAPTC Newsl.*, **55**, 2–10.

149 Cui, H., Yu, Z., Deng, J., Gao, X., Sun, Y., and Xia, G. (2009) Introgression of bread wheat chromatin into tall wheatgrass via somatic hybridization. *Planta*, **229**, 323–330.

150 Rokka, V.M., Xu, Y.S., Kankila, J., Kuusela, A., Pulli, S., and Pehu, E. (1994) Identification of somatic hybrids of dihaploid *Solanum tuberosum* lines and *S. brevidens* by species specific RAPD patterns and assessment of disease resistance of the hybrids. *Euphytica*, **80**, 207–217.

151 Nouri-Ellouz, O., Gargouri-Bouzid, R., Sihachakr, D., Triki, M.A., Ducreux, G., Driraf, N., and Lakhoua, L. (2006) Production of potato intraspecific somatic hybrids with improved tolerance to PVY and *Pythium aphanidermatum*. *J. Plant Physiol.*, **163**, 1321–1332.

152 Sjödin, C. and Glimelius, K. (1989) Transfer of resistance against *Phoma lingam* to *Brassica napus* by asymmetric somatic hybridization combined with toxin selection. *Theor. Appl. Genet.*, **78**, 513–520.

153 Mendes, B.M.J., Mourão Filho, F.A., Farias, P.M., and Benedito, V.A. (2001) Citrus somatic hybridization with potential for improved blight and CTV resistance. *In Vitro Cell. Dev. Biol. Plant*, **37**, 490–495.

154 Inoue, K., Kato, T., Nobukunib, A., Kunitake, H., and Yabuya, T. (2006) Characterization of tetraploid plants regenerated via protoplast culture of *Iris fulva* and their crossability with Japanese irises. *Sci. Hort.*, **110**, 334–335.

155 Jain, R.K., Khehra, G.S., Lee, S.H., Blackhall, N.W., Merchant, R., Davey, M.R., Power, J.B., Cocking, E.C., and Gosal, S.S. (1995) An improved procedure for plant regeneration from indica and japonica rice protoplasts. *Plant Cell Rep.*, **14**, 515–519.

156 Kaur, D., Gill, R., Sindhu, A.S., and Gosal, S.S. (1999) Protoplast culture and plant regeneration in basmati rice. *Crop Improv.*, **26**, 14–18.

157 Liu, F., Ryschka, U., Marthe, F., Klocke, E., Schumann, G., and Zhao, H. (2007) Culture and fusion of pollen protoplasts of *Brassica oleracea* L. var. Italica with haploid mesophyll protoplasts of *B. rapa* L. ssp. pekinensis. *Protoplasma*, **231**, 89–97.

158 Havkin-Frenkel, D., Dorn, R., and Leustek, T. (1997) Plant tissue culture for production of secondary metabolites. *Food Technol.*, **51**, 56–61.

159 Varindra, Saikia, S., Randhawa, R., Bajaj, K.L., and Gosal, S.S. (1997) Capsaicin accumulation in callus culture and whole fruits of *Capsicum annum*. *Plant Tissue Cult.*, **7**, 47–51.

160 Fischer, R., Emans, N., Schuster, F., Hellwig, S., and Drossard, J. (1999) Towards molecular farming in the future: using plant-cell-suspension cultures as bioreactors. *Biotechnol. Appl. Biochem.*, **30**, 109–112.

161 Srivastava, S. and Srivastava, A.K. (2007) Hairy root culture for mass-production of high-value secondary metabolites. *Crit. Rev. Biotechnol.*, **27**, 29–43.

162 Ford, C.S., Jones, N.B., and Staden, J.V. (2000) Optimization of a working cryopreservation protocol for *Pinus patula* embryogenic tissue. *In Vitro Cell. Dev. Biol. Plant*, **36**, 366–399.

163 Panis, B., Swennen, R., and Engelmann, F. (2001) Cryopreservation of plant germplasm. *Acta Horti.*, **560**, 79–86.

164 Volk, G.M. and Casperson, A.M. (2007) Plasmolysis and recovery of different cell types in cryoprotected shoot tips of *Mentha piperita*. *Protoplasma*, **231**, 215–226.

165 Towill, L.E., Bonnart, R., and Volk, G.M. (2006) Cryopreservation of *Arabidopsis thaliana* shoot tips. *Cryo Lett.*, **27**, 353–360.

166 Moukadiri, O. and Cornejo, M.J. (1996) Freezing stress response in progenies of rice plants regenerated from cryopreserved cells. *Biotechnol. Appl.*, **13**, 31.

167 Halmagyi, A., Deliu, C., Coste, A., Keul, M., Cheregi, O., and Cristea, V. (2004) Vitrification of potato shoot tips for germplasm cryopreservation. *Contrib. Botanice.*, **39**, 187–193.

168 Zhao, M.A., Xhu, Y.Z., Dhital, S.P., Khu, D.M., Song, Y.S., Wang, M.Y., and

Lim, H.T. (2005) An efficient cryopreservation procedure for potato (*Solanum tuberosum* L.) utilizing the new ice blocking agent, Supercool X1000. *Plant Cell Rep.*, **24**, 477–481.

169 Yoon-Ju, W., Kim-Haeng, H., Cho-Eun, Gi., Ko-Ho, C., Hwang-Hae, S., Park-Young, E., and Engelmann, F. (2007) Cryopreservation of cultivated and wild potato varieties by droplet vitrification procedure. *Acta Hort.*, **760**, 203–208.

170 Ding, F., Jin, S., Hong, N., Zhong, Y., Cao, Q., Yi, G., and Wang, G. (2008) Vitrification–cryopreservation, an efficient method for eliminating *Candidatus liberobacter asiaticus*, the citrus Huanglongbing pathogen, from *in vitro* adult shoot tips. *Plant Cell Rep.*, **27**, 241–250.

171 Gupta, S. and Reed, B.M. (2006) Cryopreservation of shoot tips of blackberry and raspberry by encapsulation–dehydration and vitrification. *Cryo Lett.*, **27**, 29–42.

172 Yang, N.S. and Christou, P. (1994) *Particle Bombardment Technology for Gene Transfer*, Oxford University Press.

173 Maqbool, S.B. and Christou, P. (1999) Multiple traits of agronomic importance in transgenic *indica* rice plants: analysis of transgene integration patterns, expression levels and stability. *Plant Mol. Breed.*, **5**, 471–480.

174 Gosal, S.S. and Gosal, S.K. (2000) Genetic transformation and production of transgenic plants, in *Plant Biotechnology: Recent Advances* (ed. P.C. Trivedi), Panima Publishers, New Delhi, pp. 29–40.

175 Ahmad, A., Maqbool, S.B., Riazudin, S., and Sticklen, M.B. (2002) Expression of synthetic Cry1AB and Cry1AC genes in basmati rice (*Oryza sativa* L.) variety 370 via *Agrobacterium* mediated transformation for the control of the European corn borer (*Ostrinia nubilalis*), *In Vitro Cell. Dev. Biol. Plant*, **38**, 213–220.

176 James, C. (2009) Global status of commercialized biotech/GM crops: ISAAA Brief No. 38. Ithaca, New York.

177 Gopal, J., Minocha, J.L., and Gosal, S.S. (1998) Variability in responsive potato genotypes to *in vitro* propagation. *J. Indian Potato Assoc.*, **25**, 119–124.

178 Ozturk, G. and Yldrm, Z. (2006) The effect of various nutrient media on microtuber production in potato (*Solanum tuberosum* L.) under *in vitro* conditions. *Turk J. Field Crops*, **11** (2), 38–45.

179 Ramanand, N., Singh, N., Kureel, M.L.., and Singh, S.B. (2007) Effect of transplanting spacing on growth and yield of tissue culture raised crop of sugarcane. *Indian Sugar*, **57**, 33–36.

180 Salami, S.A., Ebadi, A., Zamani, Z., and Ghasemi, M. (2007) *In vitro* mass propagation of grapevine (*Vitis vinifera* L.) from fragmented shoot tip via direct organogenesis. *Iran. J. Agrl. Sci.*, **37**, 1141–1149.

181 Salokhe, S.S. (2007) Performance of micropropagated sugarcane seed. *Indian Sugar*, **57**, 23–27.

182 Lal, M., Singh, R.K., Srivastava, S., Singh, N., Singh, S.P., and Sharma, M.L. (2008) RAPD marker based analysis of micropropagated plantlets of sugarcane for early evaluation of genetic fidelity. *Sugar Technol.*, **10**, 99–103.

183 Rashid, M.H., Khalekuzzaman, M., Hasan, M.F., Das, R., Hossain, M.S., and Mahabbat-E-Khoda, S. (2009) Establishment of an efficient method for micropropagation of an important medicinal herb (*Scoparia dulcis* L.) from shoot tips and nodal segments. *Int. J. Sustain. Crop Prod.*, **4**, 5–9.

184 Hwang, S.C. and Ko, W.H. (2004) Cavendish banana cultivars resistant to *Fusarium* wilt acquired through somaclonal variation in Taiwan. *Plant Disease*, **88**, 580–588.

185 Bajji, M., Bertin, P., Lutts, S., and Kinet, J.M. (2004) Evaluation of drought resistance-related traits in durum wheat somaclonal lines selected *in vitro*. *Aust. J. Exp. Agri.*, **44**, 27–35.

186 Leva, A.R. and Petruccelli, R. (2007) Field performance of olive somaclones derived from somatic embryos. *Acta Hort.*, **748**, 181–189.

187 James, A.C., Peraza-Echeverria, S., Peraza-Echeverria, L., and Herrera-Valenci, V.A. (2007) Variation in micropropagated plants. *Acta Hort.*, **748**, 55–63.

188 Oh, T.J., Cullis, M.A., Kunert, K., Engelborghs, I., Swennen, R., and Cullis, C.A. (2007) Genomic changes associated with somaclonal variation in banana (*Musa* spp.). *Physiol. Plant.*, **129**, 766–774.

189 Shen, X.L., Chen, J.J., Kane, M.E., and Henny, R.J. (2007) Assessment of somaclonal variation in Dieffenbachia plants regenerated through indirect shoot organogenesis. *Plant Cell Tissue Organ Cult.*, **91**, 21–27.

190 Labbani, Z., de Buyser, J., and Picard, E. (2007) Effect of mannitol pretreatment to improve green plant regeneration on isolated microspore culture in *Triticum turgidum* ssp. durum cv. "Jennah Khetifa". *Plant Breed.*, **126**, 565–568.

191 Fish, N., Karp, A., and Jones, M.G.K. (1988) Production of somatic hybrids by electrofusion in *Solanum*. *Theor. Appl. Genet.*, **76**, 260–266.

192 Hansen, L.N. and Earle, E.D. (1995) Transfer of resistance to *Xanthomonas campestris* pv. *campestris* into *Brassica oleracea* L. by protoplast fusion. *Theor. Appl. Genet.*, **9**, 1293–1300.

193 Matsumoto, K., Vilarinhos, A.D., and Oka, S. (2002) Somatic hybridization by electrofusion of banana protoplasts. *Euphytica*, **125**, 317–324.

194 Ananthakrishnan, G., Ćalović, M., Serrano, P., and Grosser, J.W. (2006) Production of additional allotetraploid somatic hybrids combining mandarins and sweet orange with pre-selected pummelos as potential candidates to replace sour orange rootstock. *In Vitro Cell. Dev. Biol. Plant*, **42**, 367–371.

195 Przetakiewicz, J., Nadolska-Orczyk, A., Kuć, D., and Orczyk, W. (2007) Tetraploid somatic hybrids of potato (*Solanum tuberosum* L.) obtained from diploid breeding lines. *Cell. Mol. Biol. Lett.*, **12**, 253–267.

196 Zhao, Z., Hu, T., Ge, X.H., Du, X., Ding, L., and Li, Z. (2008) Production and characterization of intergeneric somatic hybrids between *Brassica napus* and *Orychophragmus violaceus* and their backcrossing progenies. *Plant Cell Rep.*, **27**, 1611–1621.

17
A Systems-Based Molecular Biology Analysis of Resurrection Plants for Crop and Forage Improvement in Arid Environments

John P. Moore and Jill M. Farrant

Resurrection plants represent a remarkable group of plants possessing vegetative tissue capable of surviving water loss to an air-dry state. Many of these species grow in semiarid and arid countries, and a rich diversity is found in Southern Africa. We review the mechanisms proposed to explain how they tolerate desiccation, highlighting the processes that protect them from metabolic disruption, oxidative damage, and mechanical stress during dehydration. Also reviewed are the molecular mechanisms associated with sensing water deficit and activating transcriptional processes associated with desiccation-induced gene expression in these species. Functional genes, which encode protective macromolecules and enzymes, and the systems approaches (transcriptomics and proteomics) utilized are also discussed. The application of resurrection plant studies in the molecular engineering of valuable crop plants and potentially providing new forages is highlighted.

17.1
Introduction

The ability of organisms to survive extreme dehydration (i.e., desiccation), referred to as anhydrobiosis (i.e., life without water), has evolved on multiple occasions in the plant, animal, fungal, and microbial kingdoms. A number of higher plants, tracheophytes, were found to be capable of tolerating desiccation and so these different species became collectively referred to as "resurrection plants" [1, 2]. The name resurrection plant originates from the observation of these plants during rehydration and denotes the seemingly miraculous manner by which the leaves and stems of a desiccated resurrection plant unfolds and regreens upon water availability.

Desiccation tolerance is generally defined as "the ability to survive drying to, or below, the absolute water content of 0.1 g H_2O g^{-1} dry mass (g g^{-1}), this being equivalent to air dryness at 50% relative humidity (20 °C) and a water potential of ≤ -100 MPa [3–5]. This definition arose from studies on anhydrobiosis in desiccation-tolerant (orthodox) seeds. The vegetative tissues of angiosperm resurrection plants dry to equilibrium of the surrounding air, and thus reach water contents of

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$\leq 0.1\,g\,g^{-1}$. However desiccation tolerance studies are usually performed under glasshouse or simulated field conditions (as opposed to the constant humidity and temperature used for seed experiments). Hence, relative humidity and temperature values recorded in resurrection plant experiments are found to be highly variable. This implies that these conditions could give rise to fluctuations in the water content of tissues supposedly dry. Air-dried seeds have been suggested to contain localized pockets of water [6, 7] and this may be equally true of resurrection plants. An additional issue to consider is that water contents at full turgor can vary among species. In order to compare water contents, scientists in the field have come to use relative water content (RWC) measurements. This measure is determined gravimetrically by oven drying at 70 °C for 48 h and then dividing the obtained value by the gravimetric water content determined at full turgor. Nevertheless, valuable information on the mechanisms employed at different stages of dehydration (measured employing these approaches) in resurrection plants has been obtained.

Vegetative desiccation tolerance in angiosperms is comparatively rare, with approximately 300–400 species being reported as desiccation tolerant. Most of these species occur predominantly in the Southern Hemisphere in arid regions of Africa, South America, and Australia [2, 8]. The phenomenon of desiccation tolerance appears to have evolved independently on a number of occasions during plant evolution [9]. Among the dicotyledonous resurrection plants, there are several members in Schrophulariaceae (approximately 32 species) and Myrothanmaceae (at least 2 species although possibly more), and in the monocotyldeons there are several representatives of the Poaceae (approximately 36 species) and Velloziaceae, Xerophytaceae (approximately 28 species) [1, 2, 10]. The different genera and species of resurrection plants are unified by their common habitat requirements. These species commonly occur in shallow soils on rocky outcrops (also known as inselbergs) in arid subtropical and tropical regions [11]. Rock inselbergs, particularly because of rainfall runoff, expose resurrection plants to frequent cycles of dehydration and rehydration during the year, although the majority of time spent in the dry state is usually in the nonrainy season, which in Southern African region is winter. The exposed nature of these outcrops requires plants growing on them to be able to tolerate desiccation under both hot and cold environmental conditions. A rich diversity of resurrection plants is found in Southern Africa, a region of significant arid and semiarid areas. Several species, including *Myrothamnus flabellifolia*, *Craterostigma plantagineum*, *C. wilmsii*, *Xerophyta viscosa*, *X. humilis*, *Eragrostis nindensis*, and *Sporobolus stapfianus*, have been intensively studied with the goal of identifying the mechanisms responsible for their remarkable tolerance ([12–14], see Figure 17.1). Apart from the strong focus on South African species, very little research has been carried out on other resurrection plants from other countries. For example, resurrection species are found in the Balkans (*Ramonda serbica*), China (*Boea hygrometrica*), Australia (*Sporobolus* and *Eragrostis* spp.), in North and Central America (*Tortula ruralis*), and in South America (*Pleurostima purpurea*) [16–18]. We review the present hypotheses regarding the mechanisms of desiccation tolerance in resurrection plants, including processes associated with protecting against metabolic, oxidative, and mechanical damage due to desiccation. We also discuss

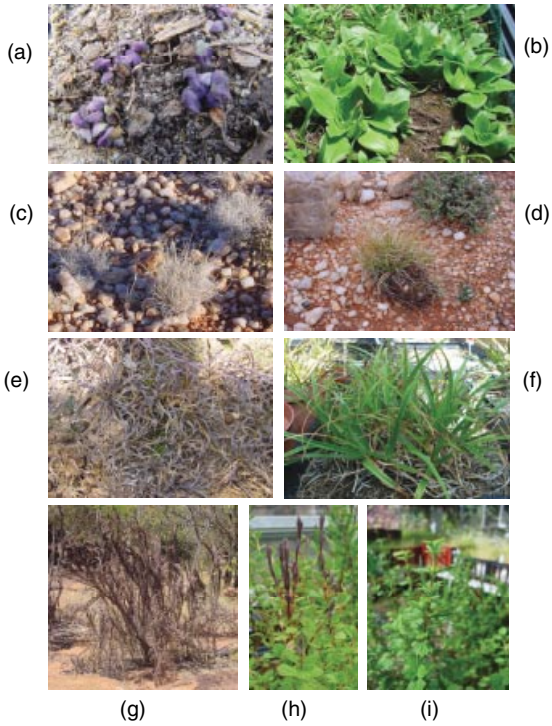

Figure 17.1 South African resurrection plants: *C. plantagineum* in the desiccated (a) and hydrated (b) state; *E. nindensis* in the dehydrated (c) and hydrated (d) state; *X. viscosa* in the desiccated (e) and hydrated (f) state; and *M. flabellifolia* in the desiccated (g), partially rehydrated, (h) and hydrated (i) state.

molecular processes associated with desiccation tolerance and the "new" systems tools that have been applied to study these plants. We conclude by highlighting the utility of research in resurrection plants as applied to improving crop and forage drought tolerance to arid environments.

17.2
Mechanisms to Alleviate Desiccation-Induced Stress

Resurrection plants are exposed to multiple stresses during dehydration and the subsequent rehydration phase. These desiccation-related stresses have been broadly classified into metabolic (including oxidative stress) and mechanical stress categories. Resurrection plants utilize common (interspecies) and unique (species-specific) strategies to alleviate or minimize the effect of these individual stresses to plant growth, reproduction, and survival. Here, we summarize the effects of these stresses on normal plant functioning and the adaptations that resurrection plants possess to counteract them.

17.2.1 Countering Oxidative and Metabolic Stresses by Modifying Photosynthesis and Sugar/Protein Metabolism

In all plants, reactive oxygen species (ROS) form as a natural consequence of metabolic processes involving electron transport [19–21]. Thus, mitochondria and chloroplasts are major sites of ROS production. Photosynthesis, in particular, is very sensitive to water deficit. Electron leakage during photosynthetic electron transport and the formation of singlet oxygen are significantly increased when cells of photosynthetic tissues suffer water loss and this has frequently been cited as a primary cause of damage and resultant plant death in most species [45]. Resurrection plants minimize ROS formation by downregulation of photosynthesis early in dehydration (from 80 to 60% relative water content) [22, 25]. Two mechanisms have evolved in resurrection plants to modify photosynthesis: these are termed poikilochlorophylly and homoiochlorophylly [22, 25].

Poikilochlorophyllous resurrection plants, usually monocotyledons, degrade their chlorophyll and dismantle their thylakoid membranes upon desiccation [22, 25]. This is a highly effective strategy to minimize ROS formation and this may explain why poikilochlorophyllous species appear to remain viable for longer periods than homoiochlorophyllous ones during desiccation [25]. The drawback of this strategy is that reassembly of the photosynthetic apparatus on rehydration requires coordinated transcription and *de novo* translation [26, 28]. Hence, poikilochlorophyllous plants require much longer periods after rehydration to resume normal growth and development. In contrast, homoiochlorophyllous species, commonly dicotyledons, retain most of their chlorophyll and thylakoid membranes intact during desiccation. They utilize various adaptations to prevent ROS production from photoactivated chlorophyll uncoupled from metabolic dissipation mechanisms (thylakoid membranes channeling excited electron to oxygen production) [22, 23, 29]. This is achieved by leaf folding and shading of inner leaves (e.g., the *Craterostigma* spp.) or adaxial surfaces (e.g., *M. flabellifolius* and *M. caffrorum*), utilizing reflective hairs and/or waxes and usually "sunscreen" pigments (anthocyanins and phenols), to reflect light back away from the leaf surface [22, 29, 30].

In addition to water-induced light stress, the progressive loss of water results in metabolic stress related to cytoplasmic crowding. The cytoplasm becomes increasingly viscous, proteins begin to denature, and membrane fusion occurs [3]. It has been proposed that desiccation-tolerant organisms counteract this stress by replacing water with compatible solutes capable of substituting for the hydrogen bonds lost due to dehydration. This water replacement hypothesis presupposes that these molecules are able to stabilize macromolecules in their native configuration during desiccation [31, 32]. Additional stabilization of the subcellular milieu is believed to be achieved via cytosolic vitrification caused by compatible solutes [3, 4, 33]. Solutes believed responsible for replacement and stabilization include (1) sucrose and oligosaccharides [5, 34, 37], (2) proteins (particularly LEA proteins and heat shock proteins) [34, 35], and (3) various compatible solutes such as proline [36]. In the case of sucrose, this is found to accumulate in the leaves and roots of all angiosperm

resurrection plants investigated thus far ([34, 37], reviewed in Ref. [15]). Sucrose is also found to be accumulated in orthodox seeds [3, 5] and this provides confirmatory evidence of an important role this sugar plays in general desiccation tolerance in plants. Recent data show that sucrose accumulates exclusively in the cytoplasmic compartments of leaf and root tissues of resurrection plants [38] and so this further confirms a possible role for this sugar in glass formation and/or stabilization of subcellular structures against desiccation. In addition to sucrose, raffinose and stachyose family oligosaccharides and trehalose have been found to accumulate upon drying in resurrection plants. Trehalose is found only to accumulate to any great extent in *M. flabellifolia*. In seeds, it is widely held that raffinose and stachyose may play an important role in cytosolic stabilization by either, or a combination of, water replacement and/or vitrification [39, 40]. These oligosaccharides are believed to function as (1) facilitators of vitrification and inhibitors of sucrose crystallization, (2) promoters of mechanical stabilization by vacuolar filling, (3) metabolic reserve polymers for excess monosaccharides (utilized in their formation), and (4) carbon sources for rehydration and recovery.

Apart from carbohydrates, proteins are also strongly implicated in desiccation protection in animals, bacteria, fungi, and plants. Particularly the late-embryogenesis abundant or LEA proteins, found at concentrations close to 4% of total cellular protein, were found to accumulate at late stages of seed development [5]. LEA proteins are found in bacteria, nematodes, and tardigrades [41] and are widely present in plants [15]. In plants, they appear to be expressed predominantly in response to desiccation, cold, drought, salt, and osmotic stress [15]. Predicted functions of LEA proteins, based on their rich hydrophilic amino acid content, include (1) water replacement and hydration buffers, (2) ion sequestration, (3) chaperonins and/or heat shock proteins, (4) antiaggregants, and (5) promoters of vitrification [5, 33, 35, 41].

17.2.2
Modulating Antioxidants and Associated Metabolism

Resurrection plants upregulate housekeeping antioxidants, so called because they are present in all plants and are crucial to maintain cellular homeostasis under day-to-day conditions and provide protection against a myriad of abiotic and biotic stresses in order to quench reactive oxygen species. However, they appear to have the additional capacity to maintain function of these antioxidants in the desiccated state and/or quickly resynthesize them upon rehydration. Furthermore, they possess the ability to produce, *de novo*, antioxidants that commonly occur in seeds [34]. The important housekeeping antioxidants, essential for maintenance of redox homeostasis, include the polar glutathione (γ-glutamyl-cysteinylglycine) and ascorbic acid [42], the non-polar soluble tocopherols and β-carotene [43], and enzymes such as superoxide dismutase, ascorbate peroxidase, and other peroxidases, mono- and dehydroascorbate reductases, glutathione reductase, and catalase. It has been shown [14] that the enzymes retain their ability to detoxify reactive oxygen species even below relative water contents of 10%, suggesting that these proteins are stable against denaturation

and desiccation. Common practice when evaluating antioxidant status under desiccation stress is to measure the concentration of individual antioxidant molecules at different stages of dehydration and rehydration. However, the use of antioxidant concentrations alone has limitations, particularly making interpretation ambiguous due to the Gaussian response to stress observed [44]. However, what appears to be a distinguishing feature in resurrection plants is their ability to maintain antioxidant potential in the dry state such that these same antioxidants can be utilized during the early stages of rehydration, thus protecting against the reactive oxygen species associated with reconstitution of full metabolism [14, 15]. Kranner *et al*. [44] have proposed that glutathione is key to the desiccation survival in a variety of desiccation-tolerant systems [44]. It was shown that the half-cell redox potential ($E_{GSSG/2GSH}$) can be used as a marker for plant stress. The authors have demonstrated that longevity of *M. flabellofolius* in the dry state was lost after 8 months when $E_{GSSG/2GSH}$ values became more positive than −160 mV [45], correlating with earlier studies on *M. flabellifolius* [12]. Recently, it was demonstrated that viability loss associated with dry stored *C. wilmsii* (3 months) and *X. humilis* (10 months) coincided with a loss of activity of the enzymes GR, catalase and SOD, even though the $E_{GSSG/2GSH}$ values did not become more positive than −160 mV (Bajic and Farrant, unpublished). Related to glutathione metabolism is ascorbate biosynthesis, due to the Haliwell–Asada antioxidant cycle. It has shown that transcription of a recently discovered gene encoding an enzyme essential to ascorbic acid biosynthesis, in the resurrection plant *X. viscosa*, is upregulated when the plants are dried below 60% relative water content and that mRNA levels remain high in the desiccated plant and during early stages of rehydration. Ascorbate levels in roots and leaves of this plant follow the same trend [46] and we propose that elevated ascorbate levels are maintained during drying and early rehydration by a combination of *de novo* synthesis and regeneration of ascorbate [14, 46]. Further to the standard housekeeping antioxidants, resurrection plants have the ability to induce, *de novo*, antioxidants such as 1- and 2-cys-peroxiredoxins, glyoxylase I family proteins, and zinc metallothionine and metallothionine-like antioxidants in response to desiccation [14, 34, 47, 48]. Polyphenols are unusual antioxidants that have also been shown to play a role in resurrection plants [49]. Moore *et al*. [49] have shown that dry leaves of *M. flabellifolia* contain a high proportion (up to 50% of the leaf dry weight) of 3, 4, 5 tri-*O*-galloylquinic acid that acts as a potent antioxidant [49]. Although this polyphenol is predominantly located in the vacuole, it has been proposed to act as an antioxidant reservoir linked to the cytoplasmic antioxidants and function as a redox buffer [30, 49, 50].

As a final observation, it would appear that the total antioxidant potential, if one combines the extent of upregulation of antioxidant enzymes and potential polyphenol antioxidant capacity and anthocyanin protection, of the homoiochlorophyllous species (*M. flabellifolius* and *Craterostigma* spp.) is greater than that of the poikilochlorophyllous types (*Xerophyta* spp. and *E. nindesis*). This supports the contention that homoiochlorophyllous resurrection plants might need better antioxidant protection against free radical activity than the poikilochlorophyllous ones [14, 23].

17.2.3
Mechanical Stress and Adaptations of Resurrection Plant Cell Walls

There appears to be two general mechanisms employed by angiosperm resurrection plants to avoid mechanical stress: (i) active and reversible wall folding as seen in the *Craterostigma* spp. [51, 52] and (ii) increased vacuolation with water replacement in vacuoles by nonaqueous substances such as in the *Xerophyta* spp. ([23], see Figure 17.2 for ultrastructural changes to resurrection plants). Desiccation induced cell wall folding is essential for structural preservation of tissue [53] and the extent and manner of folding is species specific and depends upon the chemical composition and molecular architecture of the cell wall. A comprehensive biochemical and immunocytological investigation of leaf wall changes during drying and rehydration of *C. wilmsii* has shown that the major difference between dry and hydrated walls lay only in the hemicellulose wall fractions [51, 52].

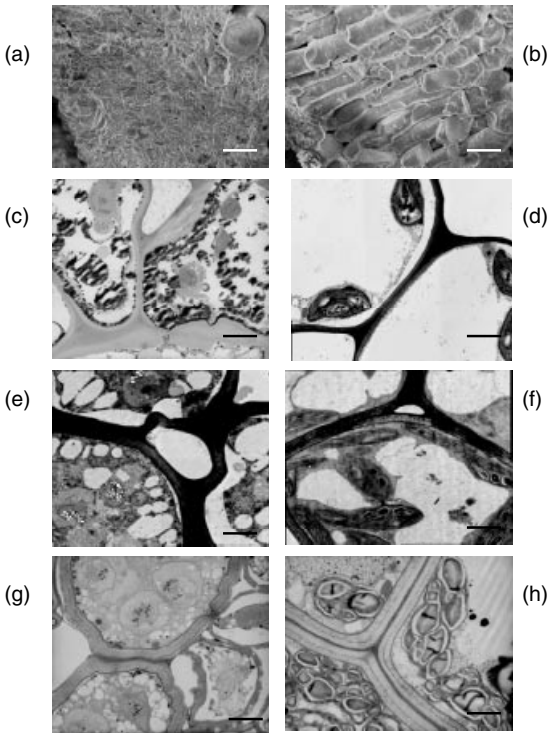

Figure 17.2 Leaf ultrastructure of South African resurrection plants in the hydrated and desiccated states. Scanning electron micrographs of flash frozen leaves *M. flabellifolia* in the desiccated (a) and hydrated (b) state. Transmission electron micrographs of *M. caffrorum* in the dehydrated (c) and hydrated (d) state, of *X. viscosa* in the desiccated (e) and hydrated (f), and of *E. nindensis* in the desiccated (g) and hydrated (h) state. Scale bars: (a) and (b) = 40 μm; (c–h) = 3 μm.

We have proposed that cleavage, or partial cleavage, of the long-chained XG units during drying into shorter, more flexible ones allows for wall folding. Ca^{2+} ions play an important role in cross-linking wall polymers, such as acid pectins, and we propose that this serves to stabilize walls in the dry state and, more importantly, prevent mechanical stress of rehydration. *C. wilmsii* is a small plant, and rehydration is rapid and if walls hydrate and unfold before cell volume is regained, plasmalemma tearing and further subcellular damage could occur [52]. Jones and McQueen-Mason [54] have shown an increase in abundance of an α-expansin transcript during drying and rehydration in leaves of *C. plantigineum* that correlated with changes in wall extensibility in that species [54]. Expansins are proposed to facilitate a complementary mechanism, whereby wall folding might be facilitated in the *Craterostigma* spp. [54].

In *M. flabellifolius* [55], wall folding occurs in the epidermis (around seemingly less flexible stomata and gland cells) and in the adjacent mesophyll cells [30]. In this species, there were no significant changes in wall components during drying, but walls contained an unusually high amount of arabinose. These arabinans were associated with pectin, presumably as arabinan polymers, and also in arabinogalactan proteins. Arabinose polymers are highly mobile and allow wall flexibility and have a high water absorbing capacity [55], which would be important for rehydration. We propose that arabinans are constitutively synthesized in leaf cell walls of *M. flabellifolius* and that their presence as "pectic plasticizers" for dehydration–rehydration cycles is crucial in this species [55].

Wall folding occurs in mesophyll cells of the grasses *E. nindensis* and *S. stapfianus*, but the biochemical nature of wall changes has not been elucidated. Interestingly, though, when tensilometry studies were performed to compare cell wall properties of the resurrection grass *E. nindensis* with three desiccation-sensitive *Eragrostis* species with differing degrees of "drought tolerance" [56], it was found that leaf tensile strength increased with degree of drought, but not desiccation tolerance [56]. Among the sensitive species, vascular bundle size and degree of lignification correlated with increased mechanical properties and water loss. However, in the resurrection species, *E. nindensis*, there was no such change in tensile properties despite the structural features of the leaves being similar to those of drought-tolerant *E. curvula* [57].

Replacement of water in vacuoles within dry tissues of resurrection plants was first suggested on the basis of ultrastructural observations that vacuoles continue to take up a large proportion of the cytoplasmic space despite the fact that there was no longer bulk water available in tissues, the remaining water being purely structure associated [14, 23, 30, 58]. A thorough biochemical analysis of *M. flabellifolius* [30, 49] revealed that the vacuoles (both hydrated and dry) contained the 3,4,5 tri-*o*-galloylquinic acid and that this polyphenolic increased upon drying to fill the vacuole and stabilize the subcellular milieu against mechanical stress. The content of vacuoles from desiccated leaves of *E. nindensis* was analyzed after nonaqueous extraction and was shown to contain proline, sucrose, and protein in equal proportions [58]. Analysis of metabolite changes in *Mohria caffrorum* showed significant fold change increases in glycerol and monohexadecanoglycerol during drying, and since these molecules are believed to be cytotoxic in large quantities, it was proposed that they accumulated

in vacuoles within the dry leaves [29]. Further studies using metabolomic technologies are underway, focused on investigating the cell wall and vacuolar content of a variety of resurrection plants.

17.3
Molecular Biology and Systems Biology of Desiccation Tolerance

A little over a decade ago, knowledge of regulatory networks controlling drought responses in plants was limited, with almost no information on signal transduction [59]. More than a decade later, while much of the signal transduction pathway in plant drought stress response has been elucidated in model plants such as *Arabidopsis*, corresponding information is still fragmentary in resurrection plants. The scarcity of information may be a consequence of the fact that of the 300–400 known resurrection plants, the molecular genetic basis of desiccation tolerance has been studied in relatively few species, namely, the mosses *S. lepidophylla* [60] and *T. ruralis* [61], the monocots *S. stapfianus* [62], *X. viscosa* [22], and *X. humilis* [48], and the dicot *C. plantagineum* [13]. In *C. plantagineum*, the synthesis of phospholipid-based signaling molecules is known as one of the earliest events in the perception of water stress. Phospholipase D (PLD) activity is induced within minutes by dehydration stress, but not by ABA [63]. PLD catalyzes the hydrolysis of phospholipids to form phosphatidic acid (PA), which in turn can regulate other components of the stress signaling pathway such as protein kinases or small GTP binding proteins. Two PLD genes, *CpPLD-1* and *CpPLD-2*, have been isolated from *C. plantagineum* [63]. The constitutively expressed *CpPLD-1* transcript is thought to be involved in early responses to dehydration by producing PA as a second messenger that transmits the stress signal, while the dehydration-induced *CpPLD-2* may be involved in phospholipid metabolism and in the rearrangement of lipids within membranes that occurs as a result of desiccation. While little is known about the early events in the perception of desiccation within resurrection plants, much more is known about downstream events in the dehydration response signaling pathway, with many genes having been characterized. Genes that are expressed in response to drought stress in resurrection plants are classified into two main types: those that control the expression of other genes such as transcription factors and regulatory RNAs, and those that encode products with putative protective functions.

17.3.1
Transcription Factors: the Magic Bullet for Plant Desiccation Tolerance?

Several classes of drought-induced transcription factors have been isolated from *C. plantagineum*; namely, MYBs, homeodomain-leucine zipper (HD-Zip) family, and a novel zinc finger ([63–67], see Table 17.1). Two Myb-related genes, *CpMyb7* and C*pMyb10*, show differential expression and regulation in response to desiccation and ABA in different tissues of *C. plantagineum* [67]. *CpMYB10* transcription is also induced by cycloheximide treatment, suggesting that *CpMYB10* expression is

Table 17.1 Functional studies and overexpression experiments performed on a number of regulatory genes from resurrection plants.

Gene	Source	Functional studies
CpMyb7 and CpMyb10	*C. plantagineum*	Show differential expression and regulation in response to desiccation and abscisic acid in different tissues of *C. plantagineum*. *CpMYB10* transcription is induced by cycloheximide treatment, expression is repressed under watered conditions, and binds to the promoter of *CDeT11-24* (a possible LEA target gene)
CpHB-7	*C. plantagineum*	The HD-Zip transcription factor family act in different pathways of the dehydration response; some mediated by abscisic acid, while others are independent of abscisic acid
CpR18	*C. plantagineum*	The C_3H-type zinc finger transcription factor CpR18 binds to a 29-bp promoter region of the LEA-like *CDeT27-45* gene that is essential for transcriptional activation by abscisic acid in *C. plantagineum*.
CpbZIP1	*C. plantagineum*	A bZIP transcription factor (CpbZIP1) and three highly conserved histone H3 proteins have been shown to bind to the promoter of the dehydration-induced group 4 LEA gene, *CpC2*. A repressor function, possibly by inhibition of other transcription factors from binding to the *CpC2* promoter, has been suggested

CDT-1	*C. plantagineum*	It was also shown that *CDT-1* has the ability to code for an siRNA and that it directs the synthesis of a double-stranded 21-bp siRNA necessary for induction of desiccation tolerance	

Gene	Source	Target	Results
CpHB-7 *Homeodomain leucine zipper* transcription factor	*C. plantagineum*	Tobacco	Overexpression resulted in early germination, increased growth rate, reduced abscisic acid sensitivity, germination sensitivity, and stomatal closure. Transgenic plants show increased salt tolerance
		Arabidopsis	Overexpression resulted in early germination, reduced abscisic acid sensitivity, stomatal closure
CpMYB10 *Myb* transcription factor	*C. plantagineum*	*Arabidopsis*	Overexpression resulted in abscisic acid hypersensitivity, glucose insensitivity, and drought and salt tolerance
CDT-1 Regulatory RNA	*C. plantagineum*	*C. plantagineum*	Constitutive desiccation tolerance in callus tissue. abscisic acid insensitive
XvSAP1 unknown	*X. viscosa*	*Arabidopsis*	Overexpression resulted in improved salinity, osmotic, and high-temperature stress

repressed under watered conditions by other (unknown) regulatory factors or that *CpMYB10* transcript is protected from degradation by inhibition of labile ribonucleases [67]. Transgenic *Arabidopsis* plants overexpressing C*pMyb10* displayed increased tolerance to drought and salt stress [68]. Interestingly, these plants also showed ABA hypersensitivity and glucose insensitivity, suggesting that C*pMyb10* is involved in mediating ABA and glucose signaling responses in *Arabidopsis* as well as the response to drought stress. Recombinant CpMYB10 protein is able to bind to specific MYB response elements within the promoter of *CDeT11-24* (a possible LEA target gene) and to elements within its own promoter, suggesting that CpMYB10 may autoregulate its expression. Several drought-regulated HD-Zip genes have been isolated from *C. plantagineum*, with some being inducible by both dehydration and exogenously applied ABA, while others are induced by dehydration stress only [64, 69]. These results demonstrate that members of the HD-Zip transcription factor family act in different pathways of the dehydration response; some are mediated by ABA, while others are independent of ABA. In both desiccation-tolerant and desiccation-sensitive plants, the expression of drought-responsive genes is mediated by both ABA-independent and ABA-dependent signal transduction pathways. Transgenic tobacco and *Arabidopsis* plants that ectopically express the HD-Zip transcription factor, *CpHB-7*, display reduced sensitivity toward ABA during seed germination and stomatal closure [65]. This suggests that CpHB-7 modifies ABA-responsive gene expression in these transgenic plants at the transcriptional level as a negative regulator. The ABA- and dehydration-responsive dehydrin gene, *CDeT6-19*, has been identified as one of the potential target genes of CpHB-7 [65]. The C_3H-type zinc finger transcription factor CpR18 binds to a 29-bp promoter region of the LEA-like *CDeT27-45* gene that is essential for transcriptional activation by ABA in *C. plantagineum* [66]. CpR18 contains an acidic SAP domain and two conserved motifs, one of which is rich in basic residues and is predicted to form a helix–loop–helix structure. CpR18 is capable of activating reporter gene expression in tobacco protoplasts by binding to the AGCCC element within the CDeT27-45 promoter. A bZIP transcription factor (CpbZIP1) and three highly conserved histone H3 proteins have been shown to bind to the promoter of the dehydration-induced group 4 LEA gene, *CpC2* [70]. *CpbZIP1* belongs to the group S of bZIP genes possessing a conserved small upstream open reading frame within the 5′-leader sequence that may be involved in regulating gene expression at the translational level. Although the exact function of CpbZIP1 is unknown, a repressor function, possibly by inhibition of other transcription factors from binding to the *CpC2* promoter, has been suggested. Two of the CpHistone H3 proteins are constitutively expressed histone H3 variants that are thought to regulate gene expression through histone modification.

17.3.2
A Role for Small RNAs as Regulators

Research over the last few years has highlighted the significance of small RNAs in regulating plant responses to abiotic stress [71]. Furini *et al.* [72] characterized the dehydration- and ABA-inducible gene *CDT-1* from *C. plantagineum* [72]. Overexpres-

sion of *CDT-1* resulted in constitutive expression of dehydration- and ABA-responsive transcripts and contributed to desiccation tolerance of *C. plantagineum* callus tissue in the absence of ABA treatment. The *CDT-1* gene lacks significant open reading frames, and appears to be naturally expressed in both sense and antisense orientations. *CDT-1* and other functionally related gene members [73] have features of short interspersed retrotransposon elements, and are hypothesized to act as regulatory noncoding RNA molecules. Recently, it has been shown that translation of the *CDT-1* transcript is not required for the induction of desiccation tolerance since *C. plantagineum* calli transformed with mutated versions of the *CDT-1* gene are constitutively desiccation tolerant [74]. The information necessary for induction of desiccation tolerance is contained within the 488-bp region at the 3′ end of the *CDT-1* gene. It was also shown that *CDT-1* has the ability to code for a small interfering RNA (siRNA) and that it directs the synthesis of a double-stranded 21-bp siRNA. Expression of the siRNA is capable of inducing desiccation tolerance in callus tissue of *C. plantagineum*. Thus, it has been demonstrated that retrotransposons and siRNA play a role in the evolution of desiccation tolerance in *C. plantagineum*. Whether this is also the case in other resurrection plants remains to be seen. *CDT-1* and its closely related gene members appear to be unique to *C. plantagineum* [73]. However, it is likely that more regulatory RNAs involved in desiccation tolerance will be discovered. Regulation of gene expression by small RNAs allows plants the flexibility to modulate stress responses and adapt to diverse and extreme habitats [71].

17.3.3
Functional Genes Encoding for LEA Proteins

The ability of resurrection plants to survive desiccation depends on the accumulation of protective proteins such as the late-embryogenesis abundant proteins. LEA proteins have been classified into 18 superfamilies on the basis of sequence homology [75]. Members of some LEA superfamilies, notably group 3 and group 4, share strong conservation of structural motifs across diverse plant species including resurrection and desiccation-sensitive plants. Many of these LEA genes are activated under mild dehydration stress conditions (>65% RWC) in both desiccation-sensitive and -tolerant tissues, and are proposed to protect plants only at higher water contents [34]. However, only genes belonging to LEA-1, -6, and -9 superfamilies are significantly expressed during seed development. These LEA genes are thought to play a role in defense against severe water loss such as that experienced by orthodox seeds during maturation or by resurrection plants during desiccation [34]. Several protective functions have been predicted for LEA proteins including a role in the unwinding or repair of DNA, forming cytoskeletal filaments to counteract the physical stresses imposed by desiccation, and acting as molecular chaperones [76]. Alternatively, LEA proteins, possibly in combination with compatible solutes and/or sugars, may replace water during desiccation and thus maintain the hydration shell of proteins and other molecules. Recently, it has been shown that LEA proteins can act synergistically with sugars such as trehalose to prevent protein aggregation during desiccation [41]. The expression of at least 16 different LEA genes,

representing seven LEA superfamilies, is activated during desiccation in *X. humilis* leaves [48]. The concurrent induction of multiple LEA genes during dehydration suggests that these LEAs may interact to stabilize and protect other proteins and membranes, or that different LEAs are specifically targeted at different organelles or cellular structures to exert their protective function. Two LEA proteins from *C. plantagineum*, CDeT11-24 and CDeT6-19, have recently been shown to be phosphorylated in both leaf and root tissues during desiccation [77]. Phosphorylation is believed to be required for the function of these proteins that is proposed to protect the catalytic activities of enzymes by altering the stability or specificity of protein–protein interactions [77]. LEAs may also play a role in recovery during rehydration in *T. ruralis* [61]. It has been proposed that in rehydrating *T. ruralis* gametophytes, LEA proteins may stabilize membranes or perhaps function in the transport of lipids for reconstitution of damaged membranes [78].

17.3.4
Functional Genes Encoding for Antioxidant Enzymes

Antioxidant enzymes such as superoxide dismutase, glutathione reductase, and ascorbate peroxidase are considered as general "housekeeping" protectants because they are not only expressed specifically in resurrection plants but are also induced in response to various abiotic stresses in desiccation-sensitive organisms [34]. However, there are some novel antioxidants that appear to be specific to desiccation tolerance. Several members of the aldehyde dehydrogenase (ALDH) have been identified both in seeds during maturation drying and in dehydrated vegetative tissues of resurrection plants. Velasco *et al.* [79] demonstrated that mRNA and protein levels of GAPDHc, a member of the ALDH11 (GAPDH) family, increased in response to desiccation in the leaves of *C. plantagineum* [77]. Recently, Kirch *et al.* [80] showed that the ABA- and dehydration-induced Cp-ALDH protein, an ALDH3 protein family homologue from *C. plantagineum*, was capable of oxidizing toxic nonanal, propionaldehyde, and acetaldehyde [80]. In *T. ruralis*, the *ALDH21A1* gene is thought to play an important role in the detoxification of aldehydes generated in response to desiccation and salinity stress, and to oxidize a novel class of stress-induced aldehydes [81]. Another antioxidant enzyme, the seed-specific 1-cys-peroxiredoxin, has been previously shown to be abundantly expressed during desiccation in the leaves of *X. humilis* and *X. viscosa* [47]. Interestingly, a 1-cys peroxiredoxin is also expressed not only during dehydration but also during rehydration of *T. ruralis* [82].

17.3.5
Functional Proteomic Studies of Desiccation Tolerance

Transcriptomic approaches have identified many mRNAs that are induced by dehydration and encode proteins that play important roles in desiccation tolerance in resurrection plants. However, there have been few studies of the proteome of resurrection plants during dehydration and/or rehydration (see Table 17.2). The importance of proteomic studies is highlighted by the fact that, in plants, there only

Table 17.2 Transcriptomic and proteomic studies performed on a variety of resurrection plants to elucidate mechanisms of their tolerance to desiccation.

Division	Species	Transcriptomic and/or proteomic study
Bryophyte	*T. ruralis*	Approximately 10 000 ESTs were obtained from drying and rehydrating gametophytes, including LEA genes involved in protection and repair [61, 86]
	T. ruralis	cDNA libraries enriched with ESTs from slow drying and rehydrating gametophytes revealed genes involved in metabolic recovery, signaling, proteosomal processing, and splicing [94]
Lycophyte	*Selaginella lepidophylla*	EST library corresponding to approximately 800 genes compared to 1300 genes from desiccation-sensitive *S. moellendorfii*. Genes identified that are involved in transport, cytoskeleton, phenylpropanoid biosynthesis, LEA proteins, ELIPs, and HSPs [60]
Angiosperm	*S. stapfianus*	144 cDNA upregulated genes in dried leaves; mostly LEA genes, defense, and detoxification genes [95]
	X. humilis	Normalized cDNA library (about 10 000 clones) from dried roots and leaves containing 424 sequenced and arrayed cDNA. Identification of LEA genes, antioxidant and signaling genes upregulated, and metabolism and growth genes downregulated [48]
	X. viscosa	Identification of water stress-induced photosynthesis-associated proteins, chaperones, RNA-binding protein, 2-Cys peroxiredoxin, and protein phosphatase 2C [28]
	B. hygrometrica	Water stress induces polypeptides involved in photosynthesis, glutathione metabolism, and phenolic metabolism [85]
	C. plantagineum	Enriched phosphoproteins correspond to chlorophyll a/b binding protein, rubisco, 14-3-3 proteins, HSPs, elongation factor 1, eukaryotic initiation factor [77]

appears to be approximately 50% correlation between the abundance of proteins and their corresponding mRNA levels. Similarly, a high percentage (40–55%) of drought-induced proteins do not show an increase in transcript abundance during dehydration in poplar. Thus, there is a clear need for proteomic studies in resurrection plants in order to understand the mechanisms of desiccation tolerance. A two-dimensional (2D) SDS-PAGE analysis of the *S. stapfianus* leaf demonstrated that the protein complement changes during the induction of desiccation tolerance [83]. 2D SDS-PAGE was also used to study *de novo* protein synthesis during rehydration in *T. ruralis* [84] and in *C. plantagineum* [77]. However, in all three studies, no protein identification was attempted. Recently, changes in the leaf proteomes of resurrection plants during desiccation were examined in *X. viscose* [28] and in *B. hygrometrica* [85] using quantitative 2D SDS-PAGE analysis. Several novel gene products, not previously isolated in transcriptomic studies, were identified as being upregulated during

dehydration. In detached *B. hygroscopica* leaf tissue, dehydration-induced proteins include an ABC transporter and a vacuolar H^+-ATPase that may be involved in protection against osmotic stress, a glutathione peroxidase-like protein that may be involved in oxidative stress protection, and a polyphenol oxidase that may prevent proteolytic activity [85]. In severely dehydration-stressed *X. viscosa* leaf tissue (35% RWC), proteins that increase in abundance include a chloroplast FtsH protease, GDP-mannose-3′,5′-epimerase, alcohol dehydrogenase, and protein phosphatase type 2C, VDAC1.1, and 2-cys peroxiredoxin that may be involved in antioxidant defense [28]. Of particular interest are proteins synthesized *de novo* upon dehydration (i.e., absent in hydrated tissues). These include a dnaK-type molecular chaperone, RNA binding protein, phosphopyruvate hydratase, and desiccation-related protein. A major hindrance to proteomic analysis in resurrection plants is the lack of a large genomic or EST database such as those available for organisms with completely sequenced genomes. For both *X. viscosa* [28] and *B. hygroscopica* [85], this has resulted in low success rate (~30%) in protein identification when searching against the NCBI nonredundant database. Although large EST collections (containing more than 10 000 ESTs and representing several thousand genes) are available for *T. ruralis* [86] and *X. humilis* [48], this number is probably still too low to increase the protein identification success rate significantly for proteomics study. For that, a much larger EST collection would be needed. New high-through put sequencing technology allows many more genes to be sequenced in a relatively short time (up to 100 Mbp per one 7–8 h instrument run). The technology can be adapted for gene discovery from cDNA [87] or small RNA [88] libraries.

17.4
Applications for Engineering Drought Tolerance in Crops and Forages

The aim of the study of desiccation tolerance in resurrection plants is to engineer agronomically valuable desiccation-sensitive crop plants for increased tolerance [89, 90]. Over 100 dehydration-induced genes from a number of resurrection plants have now been characterized [48]. However, very few of these genes have been introduced into desiccation-sensitive plants and tested for improving drought tolerance (see Table 17.1). The problem is that overexpressing single genes has proven a flawed strategy for producing drought-tolerant plants. Many of these transgenic plants show side effects, including, abscisic acid hypersensitivity or insensitivity, impaired germination ability, modified stomatal activity, glucose insensitivity, and constitute desiccation tolerance (see Table 17.1). Although some results have proven promising, such as plants showing improved salt, osmotic, and temperature stress, we still have a very unclear understanding of the reasons behind this (see Table 17.1). Limited information is available on the feasibility of transferring resurrection genes to a crop plant system and equally whether such genes will be useful in an agricultural scenario. Yield under drought stress is possibly a greater consideration than survivability, which is what resurrection plants are adapted for. Nevertheless, progress in crop improvement will be possible only with a multifaceted

approach using systems biology and molecular genetics (see review [15]), so that multiple high-impact "resurrection plant" genes can be coordinately expressed in model crop systems for drought tolerance. This approach requires data acquisition using genomics (the genome sequence of one or two model resurrection plants would be invaluable here), transcriptomics, proteomics, and metabolomics that needs to be coupled to multivariate data analysis methods (see review [15]). An alternative approach for resurrection plants is to employ them as forages in arid countries such as India and South Africa. Many resurrection plants are grasses and early surveys of resurrection grasses in India have identified these as potential forages [91, 92]. This strategy avoids the problem of "molecular reengineering" of established crops as here the objective is to convert an already desiccation-tolerant species into valuable forage by genetically enhancing nutritional value. Genetic engineering, tissue culture, and transformation technology are still fairly rudimentary in resurrection plants, but progress is being made [90, 93]. This strategy might pose a valuable alternative strategy for utilizing indigenous resources, such as resurrection plants, as drought-tolerant forages, because only a few genes, for enhanced nutritional value, need to be transferred. However, much more research is needed to confirm the reliability and validity of these approaches.

17.5
Concluding Remarks and Future Directions

The research to date outlined highlights not only a number of key differences among resurrection plants in their responses to desiccation but also some important similarities. As more transcriptomic, proteomic, and, in particular, metabolomic studies are conducted on these remarkable plants, it is likely that more of these commonalities will be discovered or confirmed. It is expected, for instance, that more studies will confirm the proposition that the evolution of desiccation tolerance in vegetative tissues is derived from a specific program of gene expression vital for orthodox seed development [34]. Data (J.M. Farrant, unpublished) is emerging that support the hypothesis that resurrection plants evolved through "vegetative tissue localized" activation of an existing seed desiccation program coupled to environmental regulation. Desiccation tolerance is a complex phenomenon and involves multiple factors, probably much more intricately connected than as outlined in the summary put forward in this chapter. More information is urgently needed both on pre- and posttranscriptional and -translational control mechanisms and on all protective proteins and metabolites that are critical for tolerance to emerge. A more holistic analysis of desiccation tolerance in resurrection plants is required, extending from leaves to roots and other organs, providing an integrative analysis. An integrated approach whereby genomic, transcriptomic, proteomic, and metabolomic data are collected from a few key "model" resurrection plants will allow for the prioritization of vital tolerance factors. This would allow for the engineering of multiple "high-impact" genes (regulatory or functional) using inducible expression systems into crop plants for improved tolerance. An alternative approach is to reactivate seed-

specific gene expression systems in vegetative tissues of crops, thereby "unlocking" innate tolerance processes and coupling these to environmental stress cues. It is hoped that such approaches will enable us to eventually obtain a clear picture of the desiccation tolerance phenomenon as it pertains to the vegetative tissues of angiosperm resurrection plants.

References

1 Gaff, D.F. (1971) *Science*, **174**, 1033–1034.
2 Gaff, D.F. (1977) *Oecologia*, **31**, 95–109.
3 Vertucci, C.W. and Farrant, J.M. (1995) *Seed Development and Germination* (eds J. Kigel and G. Galilli), Marcel Dekker., New York, pp. 237–271.
4 Walters, C., Pammenter, N.W., Berjak, P. *et al.* (2002) *Desiccation and Survival in Plants: Drying Without Dying* (eds M. Black and H.W. Pritchard), CAB International, Wallington, UK, pp. 263–291.
5 Berjak, P. and Pammenter, N.W. (2008) *Ann. Bot.*, **101**, 213–228.
6 Leubner-Metzger, G. (2005) *Plant J.*, **41**, 133–145.
7 Oracz, K., Bouteau, H.E., Farrant, J.M. *et al.* (2007) *Plant J.*, **50**, 452–465.
8 Alpert, P. and Oliver, M.J. (2002) *Desiccation and Survival in Plants: Drying Without Dying* (eds M. Black and H.W. Pritchard), CAB International, Wallington, UK, pp. 3–43.
9 Oliver, M.J., Wood, A.J., and O'Mahony, P. (1998) *Plant Growth Regul.*, **24**, 193–201.
10 Proctor, M.C.F. and Tuba, Z. (2002) *New Phytol.*, **156**, 327–349.
11 Porembski, S. and Barthlott, W. (2000) *Plant Ecol.*, **151**, 19–28.
12 Moore, J.P., Lindsey, G.G., Farrant, J.M. *et al.* (2007) *Ann. Bot.*, **99**, 211–217.
13 Bartels, D. (2005) *Integr. Comp. Biol.*, **45**, 696–701.
14 Farrant, J.M. (2007) *Plant Desiccation Tolerance* (eds M.A. Jenks and A.J. Wood), CAB International Press, Wallingford, UK.
15 Moore, J.P., Le, N.T., Brandt, W.F., Driouich, A., and Farrant, J.M. (2009) *Trends Plant Sci.*, **14**, 110–117.
16 Alpert, P. (2006) *J. Exp. Biol.*, **209**, 1575–1584.
17 Oliver, M.J., Tuba, Z., and Mishler, B.D. (2000) *Plant Ecol.*, **151**, 85–100.
18 Meirelles, S.T., Mattos, E.A., and da Silva, A.C. (1997) *Pol. J. Environ. Stud.*, **6**, 17–21.
19 Halliwell, B. and Gutteridge, J.M.C. (1999) *Free Radicals in Biology and Medicine* (3rd Edn), Oxford University Press, Oxford.
20 Apel, K. and Hirt, H. (2004) *Annual Review of Plant Biology*, **55**, 373–399.
21 Bailly, C. (2004) *Seed Science Research*, **14**, 93–107.
22 Sherwin, H. and Farrant, J.M. (1998) *Plant Growth Regul.*, **3**, 203–210.
23 Farrant, J.M., Lehner, A., Cooper, K. *et al.* (2009) *Plant J.*, **57**, 65–79.
24 Gaff, D.F. (1989) *Structural and Functional Responses to Environmental Stress: Water Shortage* (eds K.H. Kreeb, H., Richter, and T.M. Hinckley), SPB Academic Publishers, The Hague, pp. 255–268.
25 Tuba, Z., Proctor, M.C.F., and Csintalan, Z. (1998) *Plant Growth Regul.*, **24**, 211–217.
26 Dace, H., Sherwin, H.W., Illing, N., and Farrant, J.M. (1998) *Plant Growth Regul.*, **24**, 171–177.
27 Sherwin, H.W. and Farrant, J.M. (1996) *Ann. Bot.*, **78**, 703–710.
28 Ingle, R.A., Schmidt, U., Farrant, J.M., Mundree, S.G., and Thompson, J.A. (2007) *Plant Cell Envron.*, **30**, 435–446.
29 Farrant, J.M., Lehner, A., Cooper, K., and Wiswedel, S. (2009) *Plant J.*, **57**, 65–79.
30 Moore, J., Farrant, J.M., Brandt, W.B. *et al.* (2007) *Aust. J. Bot.*, **55**, 482–491.
31 Crowe, L.M. *et al.* (1986) *Biochim. Biophys. Acta*, **861**, 131–140.
32 Crowe, J.H. *et al.* (1998) *Annu. Rev. Physiol.*, **60**, 73–103.
33 Hoekstra, F.A. *et al.* (2001) *Trends Plant Sci.*, **6**, 431–438.

34 Illing, N., Denby, K., Collett, H., Shen, A., and Farrant, J.M. (2005) *Integr. Comp. Biol.*, **45**, 771–787.

35 Mtwisha *et al.* (2006) *Drought Adaptations in Cereals* (ed. J.M. Ribeaur), Food Products Press, The Haworth Press, Binghamton, New York, pp. 531–542.

36 Gaff, D.F. and McGregor, G.R. (1979) *Biol. Plant*, **21**, 92–99.

37 Peters, S., Mundree, S.G., Thomson, J.A., Farrant, J.M., and Keller, F. (2007) *J. Exp. Bot.*, **58**, 1947–1956.

38 Martinelli, T. (2008) *Journal of Plant Physiology*, **165**, 580–587.

39 Buitink, J., Hoekstra, F.A., and Leprince, O. (2002) *Desiccation and Survival in Plants: Drying Without Dying* (eds M. Black and H.W. Pritchard), CAB International, Wallington, UK, pp. 293–318.

40 Leprince, O. and Buitink, J. (2010) *Plant Science*, **179**, 554–564.

41 Goyal, K., Walton, L.J., and Tunnacliffe, A. (2005) *Biochem. J.*, **388**, 151–157.

42 Noctor, G. and Foyer, C.H. (1998) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **49**, 249–279.

43 Munné-Bosch, S. and Alegre, L. (2002) *Crit. Rev. Plant Sci.*, **21**, 31–57.

44 Kranner, I., Birtic, S., Anderson, K. *et al.* (2006) *Free Radical Bio. Med.*, **40**, 2155–2165.

45 Kranner, I. and Birtic, S. (2005) *Integr. Comp. Biol.*, **45**, 734–740.

46 Kamies, R., Rafudeen, M.S., and Farrant, J.M. (2010) *Plant Growth Regulation*, **62**, 203–211.

47 Mowla, S.B. *et al.* (2002) *Planta*, **215**, 716–726.

48 Collett, H., Shen, A., Gardner, M. *et al.* (2004) *Physiol. Plant.*, **122**, 39–53.

49 Moore, J.P., Ravenscroft, N., Lindsey, G.G. *et al.* (2005) *Biochem. J.*, **385**, 301–308.

50 Kranner, I., Beckett, R.P., Wornik, S. *et al.* (2002) *Plant J.*, **31**, 13–24.

51 Vicre, M., Sherwin, H.W., Driouich, A. *et al.* (1999) *J. Plant Physiol.*, **155**, 719–726.

52 Vicre, M., Lerouxel, O., Farrant, J.M. *et al.* (2004) *Physiol. Plant.*, **120**, 229–239.

53 Moore, J.P., Vicré-Gibouin, M., Farrant, J.M., and Driouich, A. (2008) *Physiol. Plant.*, **134**, 237–245

54 Jones, L. and McQueen-Mason, S. (2004) *FEBS Lett.*, **559**, 61–65.

55 Moore, J.P., Nguema-Ona, E.E., Chevalier, L.M. *et al.* (2006) *Plant Physiol.*, **141**, 651–662.

56 Balsamo, R., Vander Willigen, C., and Farrant, J.M. (2006) *Ann. Bot.*, **97**, 985–991.

57 Hedderson, N., Balsamo, R., Cooper, K., and Farrant, J.M. (2009) *S. Afr. J. Bot.*, **75**, 8–16.

58 Vander Willigen, C., Mundree, S.G., Pammenter, N.W., and Farrant, J.M. (2003) *Funct. Plant Biol.*, **30**, 281–290.

59 Ingram, J. and Bartels, D. (1996) *Annu. Rev. Plant Physiol. Mol. Biol.*, **47**, 377–403.

60 Iturriaga, G., Cushman, M.A.F., and Cushman, J.C. (2006) *Plant Sci.*, **170**, 1173–1184.

61 Oliver, M.J., Dowd, S.E., Zaragoza, J., Mauget, S.A., and Payton, P.R. (2004) *BMC Genomics*, **5**, 1–19.

62 Le, N.T., Blomstedt, C.K., Kuang, J., Tenlen, J., Gaff, D.F., Hamill, J.D., and Neale, A.D. (2007) *Funct. Plant Biol.*, **34**, 589–600.

63 Frank, W. *et al.* (2000) *Plant Cell*, **12**, 111–123.

64 Deng, X. *et al.* (2002) *Plant Mol. Biol.*, **49**, 601–610.

65 Deng, X. *et al.* (2006) *Plant Mol. Biol.*, **61**, 469–489.

66 Hilbricht, T. *et al.* (2002) *Plant J.*, **31**, 293–303.

67 Iturriaga, G., Leyns, L., Villegas, M., Cushman, M.A.F. *et al.* (1996) *Plant Mol. Biol.*, **32**, 707–716.

68 Villalobos, M.A. *et al.* (2004) *Plant Physiol.*, **135**, 309–324.

69 Frank, W., Phillips, J., Salamini, F. *et al.* (1998) *Plant J.*, **15**, 413–421.

70 Ditzer, A. and Bartels, D. (2006) *Plant Mol. Biol.*, **61**, 643–663.

71 Phillips, J.R. *et al.* (2007) *FEBS Lett.*, **581**, 3592–3597.

72 Furini, A., Koncz, C., Salamini, F., and Bartels, D. (1997) *EMBO J.*, **16**, 3599–3608.

73 Smith-Espinoza, C.J. *et al.* (2005) *Mol. Genet. Genomics*, **274**, 364–372.

74 Hilbricht, T. *et al.* (2008) *New Phytol.*, **179**, 877–887.

75 Dure, L. (1993) *Plant J.*, **3**, 363–369.

76 Wise, M.J. and Tunnacliffe, A. (2004) *Trends Plant Sci.*, **9**, 747–757.

77 Rohrig, H. *et al.* (2006) *Plant Cell Environ.*, **29**, 1606–1617.

78 Velton, J. and Oliver, M.J. (2001) *Plant Mol. Biol.*, **45**, 713–722.

79 Velasco, R. *et al.* (1994) *Plant Mol. Biol.*, **26**, 541–546.

80 Kirch, H.H. *et al.* (2001) *Plant J.*, **28**, 555–567.

81 Chen, X. *et al.* (2002) *J. Plant Physiol.*, **159**, 677–684.

82 Oliver, M.J. (1996) *Physiol. Plant.*, **97**, 779–787.

83 Kuang, J., Gaff, D.F., Gianello, R.D. *et al.* (1995) *Aust. J. Plant Physiol.*, **22**, 1027–1034.

84 Oliver, M.J. (1991) *Plant Physiol.*, **97**, 1501–1511.

85 Jiang, G., Wang, Z., Shang, H. *et al.* (2007) *Planta*, **225**, 1405–1420.

86 Oliver, M.J. *et al.* (2004) *BMC Genomics*, **5**, 89–108.

87 Margulies, M., Egholm, M., Altman, W. *et al.* (2005) *Nature*, **437**, 376–380.

88 Henderson, I., Zhang, X., Lu, C. *et al.* (2006) *Nat. Genet.*, **38**, 721–725.

89 Toldi, O., Tuba, Z., and Scott, P. (2009) *Plant Sci.*, **176**, 187–199.

90 Toldi, O., Tuba, Z., and Scott, P. (2010) *Rom. Biotechnol. Lett.*, **15**, 3–11.

91 Gaff, D.F., Blomstedt, C.K., Neale, A.D. *et al.* (2009) *Funct. Plant Biol.*, **36**, 1–11.

92 Gaff, D.F. and Bole, P.V. (1986) *Oecologia*, **71**, 159–160.

93 Ncanana, S., Brandt, W., Lindsey, G.G., and Farrant, J.M. (2005) *Plant Cell Rep.*, **24**, 335–340.

94 Oliver, M.J., Hudgeons, J., Dowd, S. *et al.* (2009) *Physiol. Plant.*, **136**, 437–460.

95 Neale, A., Blomstedt, C.K., Bronson, P. *et al.* (2000) *Plant Cell Environ.*, **23**, 265–277.

Section IIC Other Approaches

Improving Crop Resistance to Abiotic Stress, First Edition.
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18
Molecular Breeding for Enhancing Abiotic Stress Tolerance Using Halophytes

Ajay Parida, Suja George, and K. Kavita

Halophytes are plants that survive in environments where the salt concentration is around 200 mM NaCl. The tolerance of halophytes to salinity relies on controlled uptake and compartmentalization of Na^+ and the synthesis of compatible solutes. Identification and isolation of novel genes by genomic approaches will advance the understanding of mechanisms of high salt tolerance. Plant stress tolerance can be improved by manipulating stress-associated genes and proteins and/or overexpression of stress-associated metabolites. Here, we discuss the role of possible genes in stress mitigation and tolerance in halophytes and their overexpression to generate stress-tolerant crop plants.

18.1
Introduction

Abiotic stress limits crop productivity and their effect on plants in both natural and agricultural settings is a topic that is receiving increasing attention. Salt and drought are the two major abiotic stresses causing yield losses in crop plants. Seven percent of the land's surface and five percent of cultivated lands are affected by salinity [1], with salt stress being one of the most serious environmental factors limiting the productivity of crop plants [2]. Extensive research in plant salt tolerance has been carried out, with the aim of improving the tolerance of crop plants. Salt tolerance is the ability of plants to grow and complete their life cycle on a substrate that contains high concentrations of soluble salt. Plants have been categorized into halophytes and glycophytes depending upon their behavior in saline environments [1]. Plants that can survive on high concentrations of salt in the rhizosphere and grow well are called halophytes. Depending on their salt-tolerating capacity, halophytes are either obligate or characterized by low morphological and taxonomical diversity with relative growth rates increasing up to 50% sea water or facultative and found in less saline habitats along the border between saline and nonsaline upland and characterized by broader physiological diversity that enables them to cope with saline and nonsaline conditions [3].

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Classification of halophytes has been based on the characteristics of natural saline habitats [3, 4], the chemical composition of the shoots [5], or the ability to secrete ions [6]. Saline habitats do differ in many regards (e.g., soil water content) and differences do exist among species in the balance of Na^+ and K^+ in shoot tissues [7]. The halophytes have been discussed over the past three decades including review on general physiology of halophytes [1, 8], ecophysiology [4, 6, 9–11], photosynthesis [12], response to oxidative stress [13], flooding tolerance, and salinity tolerance [14].

18.1.1
Halophytes and their Adaptations to Salinity

Halophytes have the capacity to tolerate extreme salinity because of very special anatomical and morphological adaptations or avoidance mechanisms [15]. Sodium secretion through specialized cells is a strategy used by many halophytic plants [16]. Salts may, however, also be released through the cuticle or in guttation fluid. In addition, they may be retransported back to the roots and soil via the phloem or become concentrated in salt hairs [17]. Halophytes become succulent in response to increasing salinity, and such influential changes seem to integral to halophytic development [3]. Dropping off salt-saturated organs also removes large quantities of salt from some halophytes [18].

Halophytes utilize various physiological and biochemical mechanisms that include (1) exclusion of Na^+ at the soil root boundary and therefore from all tissues; (2) exclusion of Na^+ from the xylem and therefore from leaf, thus preventing disruption of photosynthesis; (3) inclusion of Na^+ and synthesis of compatible solutes to maintain osmotic adjustment; (4) inclusion of Na^+ and its subsequent sequestration in vacuoles [19]; and (5) inclusion of Na^+ and its eventual elimination through secretion by leaves [20].

Halophytes store about 90% of Na^+ in the shoot, at least 80% in the leaves, while the root system has a much lower Na^+ concentration compared to the aerial parts of the plant [20]. It has been found that although halophytes accumulate large amounts of Na^+ in the cells, the concentration of NaCl in the cytosol is maintained within nontoxic limits by the compartmentation of NaCl in vacuoles [21]. Induction of the CAM pathway, which increases water use efficiency, is also reported in some halophytes [22]. However, individual halophytes utilize different salt tolerance traits in different situations. Since a wide range of salt tolerance mechanisms are employed by halophytes, the precise combination of one or more mechanisms used to tolerate salinity varies between and across species and makes the study of salinity tolerance more complex.

18.1.2
Halophytes as a Source for Gene Mining

The mechanism of salinity tolerance is a very complex phenomenon. Studies have shown that components of various pathways are involved in imparting the salinity tolerance to the plants. Understanding the molecular basis of the salt stress signaling

and tolerance mechanisms is essential to breeding and genetic engineering of salt-tolerant plants [23]. Genetically engineering the plants by introducing and/or overexpressing selected genes seems to be a viable option to hasten the breeding of "improved" plants, while the introgression of genomic portions (QTL) involved in stress tolerance often brings along undesirable agronomic characteristics from the donor parents. Intuitively, genetic engineering would be a faster way to insert beneficial genes than through conventional or molecular breeding. Also, it would be the only option when genes of interest originate from cross barrier species, distant relatives, or from nonplant sources. Attempts on plant stress tolerance can be made by manipulating stress-associated genes and proteins and/or overexpression of stress-associated metabolites that would confer increased tolerance to salt or drought.

Halophytic plants are a very important genetic resource for the isolation of novel promoters and/or genes that are involved in the adaptation to salinity that could be transferred to salt-sensitive glycophytes. Identification and isolation of novel genes by genomic approaches will advance the understanding of these mechanisms [24]. Hence, different efforts in large-scale EST sequencing and analysis have been made in a number of dicotyledonous halophytes, such as *Suaeda salsa* [25], *Thellungiella halophila* [26], *Mesembryanthemum crystallinum* [27], *Aegiceras corniculatum* [28], *Avicennia marina* [29], *Tamarix hispida* [30], and *Limonium sinense* [31], and monocotyledonous halophytes such as *Leymus chinensis* [32], *Puccinellia tenuiflora* [33], *Lolium temulentum* [34], *Aeluropus littoralis* [35], and *Spartina alterniflora* [36].

This chapter summarizes the involvement of various genes in sensing and response to salt stress in halophytes according to their putative functions such as (1) genes for reestablishing ionic homeostasis or preventing damage, (2) genes with an osmotic or unknown protective function, and (3) genes for signal transduction. This paper focuses on the different genes explored from halophytes for the genetic enhancement of crop plants for abiotic stress tolerance using transgenic approach.

18.2
Genes for Reestablishing Ionic Homeostasis/Preventing Damage

Excessive intracellular or extracellular Na^+ triggers a cytoplasmic Ca^{2+} signal that involves an SOS pathway. This leads to enhanced expression of transporters for ions such as Na^+, K^+, and H^+. Na^+/H^+ antiporter located in the plasma membrane excludes Na^+ from the cells [37]. The compartmentalization of Na^+ into vacuoles provides an efficient mechanism for averting the toxic effects of Na^+ in the cytosol. The transport of Na^+ into vacuoles mediated by vacuolar Na^+/H^+ antiporters is driven by the electrochemical gradient of protons. The proton-motive force generated by the vacuolar ATPase (V-ATPase) and vacuolar pyrophosphatase (V-PPase) can drive secondary transporters, such as the Na^+/H^+ antiporter and the Ca^{2+}/H^+ antiporter, as well as organic acids, sugars, and other compound transporters to maintain cell turgor.

18.2.1
Vacuolar Na^+/H^+ Antiporter

Salt-tolerant plants such as halophytes efficiently sequester Na^+ into vacuoles, to maintain low cytosolic concentrations of Na^+ and thus allow antiporter genes to be isolated from halophytes and transformed into transgenic plants. In transgenic systems, it was found that vacuolar antiporters from glycophytes and halophytes confer salt tolerance to varying limits. Genes encoding vacuole-type Na^+/H^+ antiporters have been isolated from a number of halophytes such as *Atriplex gmelini* (AgNHX1) [38], *M. crystallinum* (McNHX1) [39], *A. dimorphostegia* (AdNHX1) [40], *Chenopodium glaucum* (CgNHX1) [41], *S. salsa* (*SsNHX*1) [42], *Porteresia coarctata* (PcNHX1) [43], and *Salicornia brachiata* (SbNHX1) [44]. A recent study identified six putative vacuolar Na^+/H^+ antiporter genes in *P. euphratica* (PeNHX1–6), a salt-resistant tree species [45].

Overexpression of *SsNHX*1 [46] and *AgNHX1* [47] in rice markedly enhanced the tolerance to salt stress (300 mM NaCl). Increased Na^+/H^+ antiport activity in the transgenic plants caused larger amounts of Na^+ to be excluded into vacuoles in individual cells, thus rendering the transgenic rice plants more tolerant to salinity [46, 47]. But another study reported that overexpression of *NHX1* genes from both glycophytic (*OsNHX1*) and halophytic (*AdNHX1, CgNHX1*) species led to similar degree of salt tolerance in transgenic rice plants [41]. The better salt tolerance in halophytes might result from a different regulation system of *NHX1* genes or mechanisms other than vacuolar Na^+ pump [48].

18.2.2
Plasma Membrane Na^+/H^+ Antiporter

In addition to Na^+ influx control and vacuolar compartmentation, Na^+ efflux is also important in maintaining a low Na^+ concentration in the cytoplasm. Unlike animal cells, which have Na^+/K^+ ATPases, or fungal and perhaps some algal cells, which have Na^+ ATPases for Na^+ efflux, plant cells do not appear to contain Na^+ ATPases. In higher plants, the main mechanism for Na^+ extrusion is powered by the operation of the plasma membrane H^+ ATPase. H^+ ATPase allows the operation of plasma membrane Na^+/H^+ antiporter that couples the downhill movement of H^+ into the cell along its electrochemical gradient to the extrusion of Na^+ against its electrochemical gradient [49]. Na^+/H^+ antiporter activity has been reported to occur across the plasma membrane of *A. nummularia* [50]. In *A. thaliana* salt overly sensitive 1 (SOS1) is a plasma membrane Na^+/H^+ antiporter that retrieves and loads Na^+ ions from and into the xylem [51]. A comparison of *SOS1* transcript in unstressed plants of *A. thaliana* and *T. halophila* revealed that the two species had similar levels of *SOS1* transcript in their shoots, while *T. halophila* possessed threefold more *SOS1* transcript in its roots than *A. thaliana* under control conditions. *A. thaliana* plants that overexpress *SOS1* are more tolerant to salt because of this Na^+ retrieval [52]. In *T. halophila*, the salt-mediated induction of shoot *ThSOS1* expression coupled with high basal root *ThSOS1* expression is likely to be a crucial factor in tightly controlling the extent of shoot Na^+ accumulation [53].

18.2.3
Vacuolar Pyrophosphatase

The vacuolar H^+-PPase is a single subunit protein located in the vacuolar membrane [54]. It pumps H^+ from the cytoplasm into vacuoles with PPi-dependent H^+ transport. Theoretically, overexpression of H^+-PPase should enhance the ability to form the pH gradient between the cytoplasm and the vacuoles, resulting in a stronger proton-motive force for the Na^+/H^+ antiporter, Ca^{2+}/H^+ antiporter, and other secondary transporters. Overexpression of H^+-PPase genes from *T. halophila* (TsVP) enhanced the salt tolerance of tobacco [55] and cotton [56]. The transgenic lines had higher H^+-PPase hydrolytic activity and the plants accumulated more Na^+ under salt stress conditions [55, 56]. A comparison of promoters of vacuolar H^+-PPase genes from *T. halophila* (TsVP1) and *Arabidopsis* (AVP1) indicated that these two promoters had seven similar motifs at similar positions. But analysis of transgenic plants expressing GUS reporter gene under the control of these promoters indicated that TsVP1 promoter was responsible for strong reporter gene activity in almost all tissues except the seeds, and the activity was induced in both shoots and roots, especially in the root tips, when treated with salt stress. Such induction was not found in transgenic *Arabidopsis* with the AVP1 promoter. Deletion analysis revealed the presence of enhancer elements in TsVP1 promoter that increased gene expression levels [57]. These results point out the importance of cloning stress tolerance genes under stress-inducible promoters for improved tolerance.

18.2.4
Potassium Transporters

Salt tolerance requires not only the adaptation to sodium toxicity but also the acquisition of potassium whose uptake is affected by high external sodium concentration. Therefore, potassium transport systems involving good selectivity of potassium over sodium can also be considered an important salt tolerance determinant [58]. In some halophytes, salinity increases the K^+ concentration of the tissue. K^+ transport is mediated by K^+ channels and high-affinity K^+ transporters both in the plasmalemma and in the tonoplast of plant cells [59]. The K^+ channels and transporters may regulate Na^+ transport – either directly because they may be incompletely selective for K^+ and transport Na^+ when presented with a high Na^+ concentration or a high Na^+/K^+ ratio or indirectly because they may buffer the cell against Na^+ uptake by maintaining rigorous K^+ homeostasis.

The families in K^+ transporters include HAK/KUP/KT and HKT transporters. These transporters control K^+ uptake and K^+/Na^+ selectivity. In *M. crystallinum*, HAK-type proteins mediates the transport of K^+, Rb^+, and Cs^+ but not that of Na^+. McHAKs seem to have a role in mediating root K^+ uptake and that could be involved in plant long-distance K^+ transport through loading and/or unloading in the vasculature [60]. The capacity of HKT to mediate Na^+ uptake in some species makes it a candidate that could have a major function for maintaining or breaking ion

homeostasis under saline conditions. Unlike the HAK transporters, transporters in the HKT family display different ion selectivity and transport mechanisms.

McHKT1, isolated from *M. crystallinum*, is a potassium transporter localized in the plasma membrane of cells of both the leaves and the roots. The expression of *McHKT1* is upregulated after a sudden increase in external salinity (400 mm NaCl) [61], as is the expression of SOS1 and some HAK transporters. The decreased storage of Na^+ in the root and enhanced transport to the shoot, with the upregulation of *Mc*HKT1, suggested to contribute to storage of Na^+ in the leaves in ice plant [61]. In *Xenopus* oocytes, *Mc*HKT1 transports Na^+ and K^+ equally [61]. In *S. salsa*, *SsHKT1* transcript was developmentally controlled and significantly upregulated by K^+ deprivation and NaCl treatment suggesting its role in ion homeostasis and salt tolerance [62]. An AKT1-type K(+) channel gene from *Puccinellia tenuiflora*, a salt-tolerant plant, was found to be localized in the plasma membrane and preferentially expressed in the roots. The expression of PutAKT1 was induced by K(+)-starvation stress in the roots and was not downregulated by the presence of excess Na(+). *Arabidopsis* plants overexpressing PutAKT1 showed enhanced salt tolerance compared to wild-type plants. PutAKT1 transgenic plants also showed a decrease in Na(+) accumulation both in the shoot and in the root. It is possible that PutAKT1 is involved in mediating K(+) uptake (i) both in low- and in high-affinity K(+) uptake range and (ii) unlike its homologues in rice, even under salt stress condition [63]. Evidence from the range of studies discussed indicates that there may be considerable variation in the transporters involved in the uptake of Na^+, not only between glycophytes and halophytes but also between species of halophyte and even at different external salt concentrations. In *M. crystallinum* coordinate regulation of multiplicity of channels, transporters, symporters, and antiporters results in irreversible transport of NaCl from root to shoot, accumulation in leaves, and sequestration of Na^+ into the vacuoles of cells in the leaves and shoot [61].

18.2.5
ROS Scavengers

The accumulation of ROS during salt stress is mainly attributed to the inhibition of photosynthesis and a decline in CO_2 fixation. Some of the ROS are highly toxic and need to be detoxified rapidly. In order to control the level of ROS and protect the cells from oxidative injury, plants have developed a complex antioxidant defense system to scavenge the ROS. These antioxidant systems include various enzymes and nonenzymatic metabolites that may also play a significant role in ROS signaling in plants [64]. A number of transgenic improvements in abiotic stress tolerance have been achieved through detoxification strategy. These include transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases, and glutathione reductases. In *Bruguiera parviflora*, salt treatment preferentially enhanced both the content of H_2O_2 and the activity of ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), and superoxide dismutase (SOD), while induc-

ing a decrease in total ascorbate and glutathione (GSH^+GSSG) content and in catalase (CAT) activity [65]. Expression of cytosolic Cu/Zn SOD in *B. gymnorrhiza* was increased after NaCl treatment and also in the presence of mannitol and abscisic acid (ABA) [66]. In *A. marina*, the mRNA transcripts of Cu/Zn SOD (Sod1), catalase (Cat1) [67], and ascorbate peroxidase (AmAPX1) [68] were upregulated under both salinity and oxidative stress. Overexpression of *A. marina* cytosolic copper/zinc SOD conferred enhanced tolerance to both salt and drought treatments in transgenic rice [69].

18.2.6
Genes with an Osmotic/Protective Function

Compatible solutes or osmolytes would be essential for coordinated regulation of vacuolar and cytoplasmic volumes. Compatible solutes are nontoxic solutes that could increase in high concentrations in the cytosol and be compatible with metabolic activity. They would be important to adapt plants to drought, as they could enhance osmotic adjustment and allow turgor maintenance of cells that would otherwise dehydrate. In addition, certain solutes have a metabolic protective role. They could stabilize soluble or membrane proteins and thus maintain growth at high salinity, and the term osmoprotectant has arisen for this function. There are four main classes of solutes that could have an osmotic or protective role: N-containing solutes such as proline and glycine betaine; sugars such as sucrose and raffinose; straight-chain polyhydric alcohols (polyols) such as mannitol and sorbitol; and cyclic polyhydric alcohols (cyclic polyols) [70].

Many crops lack the ability to synthesize the special osmoprotectants that are naturally accumulated by stress-tolerant organisms. It is believed that osmoregulation would be the best strategy for abiotic stress tolerance, especially if osmoregulatory genes could be triggered in response to drought and salinity. Therefore, a widely adopted strategy has been to engineer crops with such osmolytes for abiotic stress tolerance.

18.2.7
Amines

Glycine-betaine (GB) highly accumulates as a compatible solute in certain plants and has been considered to play a role in the protection from salt stress. In plants, glycine-betaine is synthesized from choline in two steps, the first being catalyzed by choline monooxygenase (CMO) that requires phosphoethanolamine *N*-methyltransferase (PEAMT) and *S*-adenosyl-L-methionine (SAM) leading to synthesis of betaine-aldehyde, which is further oxidized by betaine-aldehyde dehydrogenase (BADH). CMO has been cloned and characterized from halophytes such as *Beta vulgaris* subsp. *maritime* (*BvCMO*) [71], *A. hortensis* (*AhCMO*) [72], *A. prostrata* (ApCMO) [73], and *A. nummularia* (*AmCMO*) [74]. CMO expression was highly induced upon salt treatment in *A. hortensis* [75] and *A. prostrata* [73]. Drought stress also induced the expression of *AhCMO*, but with ABA treatment *AhCMO* was induced only slight-

ly [75]. ABA treatment did not induce *ApCMO* in *A. prostrate* showing that *ApCMO* mRNA does not depend upon exogenous ABA [73].

Metabolic engineering of GlyBet in all the plants suffered from one feature: the GlyBet level in transformants is lower than that in the natural accumulator to adjust the osmotic pressure *in vivo* [76]. The transport of choline into the chloroplast constrains GlyBet accumulation in CMO^+ tobacco [77]. If supplied with extraneous substrate, transgenic plants would then synthesize enough GlyBet and greatly promote stress tolerance [78, 79]. Overexpression of *AhCMO* improved drought tolerance in transgenic tobacco and the transgenic plants also performed better under salt stress [75]. Transplastomic tobacco plants overexpressing BvCMO gene exhibited increased tolerance to salt and drought stress. Accumulation of GlyBet in transplastomic plants enhances the net photosynthetic rate and apparent quantum yield of photosynthesis under salt stress condition [80].

BADH gene has been isolated and characterized from halophytes such as *A. hortensis* (*AhBADH*) [81], *A. marina* (*AmBADH*) [82], and *S. liaotungensis* (*SlBADH*) [40]. Overexpression of *AhBADH* gene into rice [83], wheat [84], and white clover [85] and *SlBADH* into tobacco plants [40] improved salt tolerance. Cotargeting multiple steps in the same pathway was found to be a successful strategy for overexpressing glycine-betaines in plants. A study by Yilmaz and Bülow [86] reported that salt tolerance can be enhanced by genetic engineering of tobacco plants with the betaine aldehyde–choline dehydrogenase fusion protein.

18.2.8 Proline

Among compatible osmolytes, proline accumulates in many plants in response to abiotic stress [87]. Proline accumulation was correlated with improved plant performance under salt stress. In plants, the proline biosynthetic pathway from glutamate proceeds through the action of a determining enzyme, Δ1-pyrroline-5-carboxylate synthetase (P5CS). Proline catabolism is catalyzed by proline dehydrogenases (PDHs). Stress-induced increase in proline content is caused by concerted induction of proline biosynthesis genes and by repression of proline catabolism genes [87]. Undetectable level of PDH transcripts after NaCl stress in *T. halophila* resulted in reduced capacity for proline catabolism in *T. halophila*. Higher proline accumulation in *T. halophila* suggests that changes in *T. halophila* PDH expression could cause significant increases in free proline levels. Increased accumulation of proline leads to improved salt stress tolerance and is possibly a reason for improved salt stress tolerance of *T. halophila* in comparison to *A. thaliana* [53]. Increased levels of proline accumulation have also been observed in salt-stressed calli of *S. nudiflora* suggesting that proline protects the callus cells from membrane damage caused by free radicals formed during salt stress [88]. The ability of NaCl to improve the performance of *Sesuvium portulacastrum* under mannitol-induced water stress may be due to its effect on osmotic adjustment through Na^+ and proline accumulation, which is coupled with an improvement in photosynthetic activity [89].

18.2.9
Polyols

Accumulation of polyols, either straight-chain metabolites such as mannitol and sorbitol or cyclic polyols such as *myo*-inositol or its methylated derivatives such as pinitol is correlated with tolerance to drought and/or salinity [90]. Many naturally occurring salt or drought-tolerant plants accumulate such compounds during stress. These metabolites are considered compatible solutes that act by providing osmotic adjustment and by lowering the osmotic potential, thus increasing the water retention capacity of the plant [91]. *Myo*-inositol, the precursor of pinitol, is synthesized through L-*myo*-inositol 1-phosphate synthase (MIPS) coded by the *INO1* gene. The enzymatic product of MIPS is specifically dephosphorylated by a Mg^{++}-dependent L-*myo*-inositol 1-phosphate phosphatase to form free inositol. Inositol is methylated to pinitol by the inositol methyl transferase coded by an *IMT1* gene in an *S*-adenosyl methionine (SAM)-dependent reaction [90]. *IMT1* gene in halophytes has been cloned and characterized from *M. crystallinum* [90] and *P. coarctata* [92]. The transcript and protein content of *PcIMT1* was substantially upregulated in salinity and ABA in *P. coarctata*. The halophytic ice plant accumulates predominantly D pinitol under salinity and low-temperature stresses.

Several halophytic and nonhalophytic species are reported to contain pinitol as the major soluble carbohydrate in their leaves. The halophytic wild rice *P. coarctata* harbors a unique salt-tolerant MIPS coded by *PcINO1* that is able to generate *myo*-inositol even at high salt concentration, and the inositol pool in the plant is well maintained during salinity [93, 94]. Overexpression of *PcINO1* has been reported to confer salt-tolerant phenotype with unabated photosynthetic functions to transformed tobacco plants. In *M. crystallinum*, *McINO1* is induced upon salt stress but in *A. thaliana AtINO1* is not induced by salt stress [95]. A coordinated functioning of both the *INO1* gene and the *IMT1* gene is expected to be operative during salt stress in *M. crystallinum* [95] and *P. coarctata* [92] for synthesis of pinitol.

18.3
Genes for Signal Transduction

Candidate genes controlling growth are probably involved in signaling pathways and respond to hormones, transcription factors, protein kinases, protein phosphatases, and other signaling molecules. Transcription factors are proteins that respond to environmental stimuli through a signaling cascade and bind to specific regulatory sites upstream of constituent genes in a regulatory network by direct physical interaction or in combination with other proteins. Consequently, an alteration of the expression of transcription factor genes results in dramatic differences in the expression of multiple genes in a plant [96]. NAC proteins form a large family of plant-specific DNA binding transcription factors that are gaining importance in recent times with respect to understanding plant development and adaptation. In *A. marina*, *AmNAC1* transcript expression was upregulated by NaCl and ABA treatment [97].

The zinc finger proteins (ZPT2) have been previously used as candidates to show how a subset of transcription factors might be involved in the water stress response at the level of transcriptional regulation [98]. In *T. halophila*, *ThZF1*, encoding a plant-specific transcription factor, is induced at the transcription level by both drought and salt [99]. Overexpression of *AhDREB1* of *A. hortensis* improved the salt tolerance in transgenic tobacco through functioning as a regulatory molecule in response to salt stress [100]. Recently, a novel zinc finger gene designated AlSAP was isolated from the halophyte grass *A. littoralis*. Sequence homology analysis showed that the AlSAP protein is characterized by the presence of two conserved zinc finger domains A20 and AN1. AlSAP was found to be induced not only by various abiotic stresses such as salt, osmotic pressure, heat, and cold but also by abscisic acid and salicylic acid (SA). Tobacco plants expressing the AlSAP gene exhibited an enhanced tolerance to high-salinity stress. Moreover, the transgenic plants were able to complete their life cycle and to produce viable seeds under high salt conditions, while the wild-type plants died at the vegetative stage [101]. Such studies characterizing novel genes add on to the available information on salt tolerance mechanisms in halophytes.

18.4 Conclusions

Halophytes are a diverse group of plants with varying degrees of salt tolerance, yet they appear to share in common the ability to sequester NaCl in cell vacuoles as the major plant osmoticum. Efforts to produce salt-tolerant crops were aimed mainly at increasing the salt exclusion capacity of glycophytes. However, these efforts have not produced breakthroughs in salt tolerance [102]. Progress in producing highly tolerant crops may require a change in strategy, to attempt to introduce halophyte genes directly into glycophytes [103]. This chapter focuses on the combination of the genes that impart salt tolerance in halophytes and hence their use in the genetic engineering of salt-tolerant crops. Thus, engineering for accumulation of salt in vacuolated cells, together with the active extrusion of Na^+ from nonvacuolated cells (i.e., young and meristematic tissue) will allow the maintenance of a high cytosolic K^+/Na^+ ratio. This in combination with the enhanced production of compatible solutes will generate transgenic crop plants that can tolerate and grow in high soil salt concentrations.

References

1 Flowers, T.J., Garcia, A., Koyama, M., and Yeo, A.R. (1997) *Acta Physio. Plant.*, **19**, 427–433.

2 Ashraf, M. (1999) *Crit. Rev. Plant Sci.*, **13**, 17–42.

3 Waisel, Y. (1972) *The Biology of Halophytes*, Academic Press, London, UK.

4 Le Houérou, H.N. (1993) Salt-tolerant plants for the arid regions of the mediterranean isoclimatic zone, in *Towards the Rational Use of High Salinity Tolerant Plants* (eds H. Lieth and A. Masoon), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 403–422.

5 Albert, R., Pfundner, G., Hertenberger, G., Kastenbauer, T., and Watzka, M. (2000) The physiotype approach to understanding halophytes and xerophytes, in *Ergebnisse Weltweiter Ökologischer Forschung* (eds S.W. Breckle, B. Schweizer, and U. Arndt), Verlag Günter Heimbach, Stuttgart, Germany, pp. 69–87.

6 Breckle, S.W. (2002) Salinity, halophytes and salt affected natural ecosystems, in *Salinity: Environment–Plants–Molecules* (eds A. Läuchli and U. Lüttge), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 53–77.

7 Wang, S.M., Zheng, W.J., Ren, J.Z., and Zhang, C.L. (2002) *J. Arid Environ.*, **52**, 457–472.

8 Flowers, T.J. (1985) *Plant Soil*, **89**, 41–56.

9 Ball, M.C. (1988) *Trees*, **2**, 129–142.

10 Rozema, J. (1991) *Aquat. Bot.*, **39**, 17–33.

11 Ungar, I.A. (1991) *Ecophysiology of Vascular Halophytes*, CRC Press, Boca Raton, FL.

12 Lovelock, C.E. and Ball, M. (2002) Influence of salinity on photosynthesis of halophytes, in *Salinity: Environment–Plant–Molecules* (eds A. Läuchli and U. Lüttge), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 315–339.

13 Jithesh, M.N., Prashanth, S.R., Sivaprakash, K.R., and Parida, A.K. (2006) *J. Genet.*, **85**, 237–254.

14 Colmer, T.D. and Flowers, T.J. (2008) *New Phytol.*, **179**, 964–974.

15 Flowers, T.J., Hajibagheri, M.A., and Clipson, N.J.W. (1986) *Quart. Rev. Biol.*, **61**, 313–337.

16 Fitzgerald, M.A., Orlovich, D.A., and Allaway, W.G. (1992) *New Phytol.*, **120**, 1–7.

17 Stenlid, G. (1956) in *Encyclopedia of Plant Physiology* (ed. E. Ruhland), **vol**. 4, Springer, Berlin, pp. 615–637.

18 Chapman, M. (1968) Vegetation under saline conditions, in *Saline Irrigation for Agriculture and Forestry* (ed. H. Buyko), Dr. W. Junk Publishers, The Hague, pp. 210–216.

19 Blumwald, E., Aharon, G.S., and Apse, M.P. (2000) *Biochem. Biophys. Acta*, **1465**, 140–151.

20 Flowers, T.J., Troke, P.F., and Yeo, A.R. (1977) *Annu. Rev. Plant Physiol.*, **28**, 89–121.

21 Binzel, M.L., Hess, F.D., Bressan, R.A., and Hasegawa, P.M. (1988) *Plant Physiol.*, **86**, 507–514.

22 Bohnert, H.J., Nelson, D.E., and Jensen, R.G. (1995) *Plant Cell*, **7**, 1099–1111.

23 Chinnusamy, V., Jagendorf, A., and Zhu, J.K. (2005) *Crop Sci.*, **45**, 437–448.

24 Bohnert, H.J., Ayoubi, P., Borchert, C., Bressan, R.A., Burnap, R.L., Cushman, C., Cushman, M.A., Deyholos, M., Fischer, R., Galbraith, D.W., Hasegawa, P.M., Jenks, M., Kawasaki, S., Koiwa, H., Kore-eda, S., Lee, B.H., Michalowski, C.B., Misawa, E., Nomura, M., and Ozturk, N. (2001) *Plant Physiol. Biochem.*, **39**, 295–311.

25 Zhang, L., Ma, X.L., Zhang, Q., Ma, C.L., Wang, P.P., Sun, Y.F., Zhao, Y.X., and Zhang, H. (2001) *Gene*, **267**, 193–200.

26 Wang, Z.L., Li, P.H., Fredricksen, M., Gong, Z.Z., Kim, C.S., Zhang, C., Bohnert, H.J., Zhu, J.K., Bressan, R.A., Hasegawa, P.M., Zhao, Y.X., and Zhang, H. (2004) *Plant Sci.*, **166**, 609–616.

27 Kore-eda, S., Cushman, M.A., Akselrod, I., Bufford, D., Fredrickson, M., Clark, E., and Cushman, J.C. (2004) *Gene*, **27**, 83–92.

28 Fu, X., Huang, Y., Deng, S., Zhou, R., Yang, G., Ni, X., Li, W., and Shi, S. (2005) *Plant Sci.*, **169**, 147–154.

29 Mehta, P.A., Sivaprakash, K., Parani, M., Venkataraman, G., and Parida, A.K. (2005) *Theor. Appl. Genet.*, **110**, 416–424.

30 Gao, C., Wang, Y., Liu, G., Yang, C., Jiang, J., and Li, H. (2008) *Plant Mol. Biol.*, **66**, 245–258.

31 Chen, S.H., Guo, S.L., Wang, Z.L., Zhao, J.Q., Zhao, Y.X., and Zhang, H. (2007) *DNA Seq.*, **18**, 61–67.

32 Jin, H., Plaha, P., Park, J.Y., Hong, C.P., Lee, I.S., Yang, Z.H., Jiang, G.B., Kwak, S.S., Liu, S.K., Lee, J.S., Kim, Y.A., and Lim, Y.P. (2006) *Plant Sci.*, **170**, 1081–1086.

33 Wang, Y., Chu, Y., Liu, G., Wang, M.H., Jiang, J., Hou, Y., Qu, G., and Yang, C. (2007) *J. Plant Physiol.*, **164**, 78–89.

34 Baldwin, J.C. and Dombrowski, J.E. (2006) *Plant Sci.*, **171**, 459–469.

35 Zouari, N., Ben Saad, R., Legavre, T., Azaza, J., Sabau, X., Jaoua, M., Masmoudi, K., and Hassairi, A. (2007) *Gene*, **404**, 61–69.

36 Baisakh, N., Subudhi, P.K., and Varadwaj, P. (2008) *Funct. Integr. Genomics*, **8**, 287–300.

37 Zhu, J.K. (2003) *Curr. Opin. Plant Biol.*, **6**, 441–445.

38 Hamada, A., Shono, M., Xia, T., Ohta, M., Hayashi, Y., Tanaka, A., and Hayakawa, T. (2001) *Plant Mol. Biol.*, **46**, 35–42.

39 Chauhan, S., Forsthoefel, N., Ran, Y., Quigley, F., Nelson, D.E., and Bohnert, H.J. (2000) *Plant J.*, **24**, 511–522.

40 Li, J.Y., Zhang, F.C., Ma, J., Cai, L., Bao, Y.G., and Wang, B. (2003) *Plant Physiol. Commun.*, **6**, 585–588.

41 Li, J.Y., He, X.W., Xu, L., Zhou, J., Wu, P., Shou, H.X., and Zhang, F.C. (2008) *J. Zhejiang Univ. Sci. B.*, **9**, 132–140.

42 Ma, X.L., Zhang, Q., Shi, H.Z., Zhu, J.K., Xhao, Y.X., Ma, C.L., and Zhang, H. (2004) *Biol. Plant.*, **48**, 219–225.

43 Praseetha, K. (2007) Isolation, characterization and overexpression of a tonoplast Na^+/H^+ antiporter from the wild rice *Porteresia coarctata* in transgenic system. PhD thesis, University of Madras, Chennai, India.

44 Jha, A., Joshi, M., Yadav, N.S., Agarwal, P.K., and Jha, B. (2010) *Mol. Biol. Rep.* doi: 10.1007/s11033-010-0318-5

45 Ye, C.Y., Zhang, H.C., Chen, J.H., and Xia, X.L., and Yin, W.L. (2009) *Physiol. Plant.*, **137**, 166–174.

46 Zhao, F., Wang, Z., Zhang, Q., Zhao, Y., and Zhang, H. (2006) *J. Plant. Res.*, **119**, 95–104.

47 Ohta, M., Hayashi, Y., Nakashima, A., Hamada, A., Tanaka, A., Nakamura, T., and Hayakawa, T. (2002) *FEBS Lett.*, **532**, 279–282.

48 Xiong, L. and Zhu, J.K. (2002) Salt tolerance, The Arabidopsis Book.

49 Sussman, M.R. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 211–234.

50 Hassidim, M., Braun, Y., Lerner, Y.H.R., and Reinhold, L. (1990) *Plant Physiol.*, **94**, 1795–1801.

51 Shi, H., Ishitani, M., Kim, C., and Zhu, J.K. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 6896–6901.

52 Shi, H., Lee, B.H., Wu, S.J., and Zhu, J.K. (2003) *Nat. Biotech.*, **21**, 81–85.

53 Kant, S., Kant, P., Raveh, E., and Barak, S. (2006) *Plant Cell Environ.*, **29**, 1220–1234.

54 Maeshima, M. (2000) *Biochim. Biophys. Acta.*, **1465**, 37–51.

55 Gao, F., Gao, Q., Duan, X.G., Yue, G.D., Yang, A.F., and Zhang, J.R. (2006) *J. Exp. Bot.*, **57**, 3259–3270.

56 Lv, S., Yang, A., Zhang, K., Wang, L., and Zhang, J. (2008) *Mol. Breed.*, **20**, 233–248.

57 Sun, Q., Gao, F., Zhao, L., Li, K., and Zhang, J. (2010) *BMC Plant Biol.*, **10**, 90.

58 Rodriguez-Navarro, A. (2000) *Biochem. Biophysiol. Acta*, **1469**, 1–30.

59 Niu, X., Bressan, R.A., Hasegawa, P.M., and Pardo, J.M. (1995) *Plant Physiol.*, **109**, 735–742.

60 Su, H., Golldack, D., Zhao, C., and Bohnert, H.J. (2002) *Plant Physiol.*, **129**, 1482–1493.

61 Su, H., Balderas, E., Vera-Estrella, R., Golldack, D., Quigley, F., Zhao, C.S., Pantoja, O., and Bohnert, J.H. (2003) *Plant Mol. Biol.*, **52**, 967–980.

62 Shao, Q., Zhao, C., Han, N., and Wang, B.S. (2008) *DNA Seq.*, **19**, 106–114.

63 Ardie, S.W., Liu, S., and Takano, T. (2010) *Plant Cell Rep.*, **29**, 865–874.

64 Vranová, E., Inzé, D., and Van Breusegem, F. (2002) *J. Exp. Bot.*, **53**, 1227–1236.

65 Parida, A.K., Das, A.B., and Mohanty, P. (2004) *J. Plant Physiol.*, **161**, 531–542.

66 Takemura, T., Hanagata, N., Dubinsky, Z., and Karube, I. (2002) *Trees*, **16**, 94–99.

67 Jithesh, M.N., Prashanth, S.R., Sivaprakash, K.R., and Parida, A.K. (2006) *Plant Cell Rep.*, **25**, 865–876.

68 Kavitha, K., Venkataraman, G., and Parida, A.K. (2008) *Plant Physiol. Biochem.*, **46**, 794–804.

69 Prashanth, S.R., Sadhasivam, V., and Parida, A.K. (2008) *Transgenic Res.*, **17**, 281–291.

70 Chen, T.H. and Murata, N. (2002) *Curr. Opin. Plant Biol.*, **5**, 250–257.

71 Russell, B.L., Rathinasabapathi, B., and Hanson, A.D. (1998) *Plant Physiol.*, **116**, 859–865.

72 Shen, Y.G., Du, B.X., Zhang, J.S., and Chen, S.Y. (2001) *Sheng Wu Gong Cheng Xue Bao*, **17**, 1–6.

73 Wang, L.W. and Showalter, A.M. (2004) *Physiol. Plant.*, **120**, 405–412.

74 Tabuchi, T., Kawaguchi, Y., Azuma, T., Nanmori, T., and Yasuda, T. (2005) *Plant Cell Physiol.*, **46**, 505–513.

75 Shen, Y.G., Du, B.X., Zhang, W.K., Zhang, J.S., and Chen, S.Y. (2002) *Theor. Appl. Genet.*, **105**, 815–821.

76 Sakamoto, A. and Murata, N. (2002) *Plant Cell Environ.*, **25**, 163–171.

77 McNeil, S.D., Rhodes, D., Russell, B.L., Nuccio, M.L., Yair, S.H., and Hanson, A.D. (2000) *Plant Physiol.*, **124**, 153–162.

78 Nuccio, M.L., Russell, B.L., Nolte, K.D., Rathinasabapathi, B., Gage, D.A., and Hanson, A.D. (1998) *Plant J.*, **16**, 487–496.

79 Huang, J., Hirji, R., Adam, L., Rozwadowski, K.L., Hammerlindl, J.K., Keller, W.A., and Selvaraj, G. (2000) *Plant Physiol.*, **122**, 747–756.

80 Zhang, J., Tan, W., Yang, X.H., and Zhang, H.X. (2008) *Plant Cell Rep.*, **27**, 1113–1124.

81 Xiao, G., Zhang, G.Y., Liu, F.H., Wang, J., Chen, S.Y., Li, C., and Geng, H.Z. (1995) *Chin. Sci. Bull.*, **40**, 741–745.

82 Hibino, T., Meng, Y.L., Kawamitsu, Y., Uehara, N., Matsuda, N., Tanaka, Y., Ishikawa, H., Baba, S., Takabe, T., Wada, K., Ishii, T., and Takabe, T. (2001) *Plant Mol. Biol.*, **45**, 353–363.

83 Guo, Y., Zhang, L., Xiao, G., and Chen, S.Y. (1997) *Sci. China (SeriesC)*, **27**, 151–155.

84 Guo, B.H., Zhang, Y.M., Li, H.J., Du, L.Q., Li, Y.X., Zhang, J.S., Chen, S.Y., and Zhu, Z.Q. (2000) *Acta Bot. Sin.*, **42**, 279–283.

85 Chen, C.F., Li, Y.W., Chen, Y., Bai, J.R., Li, H., Zhu, Y.F., Chen, S.Y., and Jia, X. (2004) *Yi Chuan Xue Bao*, **31**, 97–101.

86 Yilmaz, J.L. and Bülow, L. (2002) *Biotechnol. Prog.*, **18**, 1176–1182.

87 Delauney, A.J. and Verma, D.P.S. (1993) *Plant J.*, **4**, 215–223.

88 Cherian, S. and Reddy, M.P. (2003) *Biol. Plant.*, **46**, 193–198.

89 Slama, I., Ghnaya, T., Messedi, D., Hessini, K., Labidi, N., Savoure, A., and Abdelly, C. (2007) *J. Plant Res.*, **120**, 291–299.

90 Vernon, D.M. and Bohnert, H.J. (1992) *Plant Physiol.*, **99**, 1695–1698.

91 LeRudulier, D. and Bouillard, L. (1983) *Appl. Environ. Microbiol.*, **46**, 152–159.

92 Sengupta, S., Patra, B., Ray, S., and Majumder, A.L. (2008) *Plant Cell Environ.*, **31**, 1442–1459.

93 Majee, M., Maitra, S., Dastidar, K.G., Pattnaik, S., Chatterjee, A., Hait, N.C., Das, K.P., and Majumder, A.L. (2004) *J. Biol. Chem.*, **279**, 28539–28552.

94 Ghosh-Dastidar, K., Maitra, S., Goswami, L., Roy, D., Das, K.P., and Majumder, A.L. (2006) *Plant Physiol.*, **140**, 1279–1296.

95 Ishitani, M., Majumder, A.L., Bornhouser, A., Michalowski, C.B., Jensen, R.G., and Bohnert, H.J. (1996) *Plant J.*, **9**, 537–548.

96 Liu, L., White, M.J., and MacRae, T.H. (1999) *Eur. J. Biochem.*, **262**, 247–257.

97 Ganesan, G., Sankararamasubramanian, H.M., Jithesh, M.N., Sivaprakash, K.R., and Parida, A.K. (2008) *Plant Physiol. Biochem.*, **46**, 928–934.

98 Sakamoto, H., Araki, T., Meshi, T., and Iwabuchi, M. (2000) *Gene*, **248**, 23–32.

99 Xu, S.M., Wang, X.C., and Chen, J. (2007) *Plant Cell Rep.*, **26**, 497–506.

100 Shen, Y.G., Yan, D.Q., Zhang, W.K., Du, B.X., Zhang, J.S., Liu, Q., and Chen, S.Y. (2003) *Acta Bot. Sin.*, **45**, 82–87.

101 Ben Saad, R., Zouari, N., Ben Ramdhan, W., Azaza, J., Meynard, D., Guiderdoni, E., and Hassairi, A. (2010) *Plant Mol. Biol.*, **72**, 171–190.

102 Flowers, T.J. and Yeo, A.R. (1995) *Aust. J. Plant Physiol.*, **22**, 875–884.

103 Bohnert, H.J. and Jensen, R.G. (1996) *Aust. J. Plant Physiol.*, **23**, 661–666.

19
Helicases in Improving Abiotic Stress Tolerance in Crop Plants

Narendra Tuteja, Sarvajeet Singh Gill, and Renu Tuteja

Abiotic stress conditions adversely affect plant growth and limit agricultural production worldwide. Minimizing these losses is a major area of concern for all countries. Salinity, drought, and cold are among the major environmental stresses that greatly influence the growth, development, survival, and yield of plants. Several genes including the genes for helicases are known to express under the influence of various abiotic stresses. The helicases are ubiquitous enzymes that catalyze the unwinding of energetically stable duplex DNA (DNA helicases) or duplex RNA secondary structures (RNA helicases). Most helicases are members of DEAD-box protein superfamily that play essential roles in basic cellular processes regulating plant growth and development, such as DNA replication, repair, recombination, transcription, ribosome biogenesis, and translation initiation. It seems, therefore, that DEAD-box helicase might also be playing an important role in stabilizing growth in plants under stress conditions by regulating some stress-induced pathways. There are now few reports on the upregulation of DEAD-box helicases in response to abiotic stresses. The exact mechanism of helicase-mediated tolerance of stress has not yet been understood. There could be two possible sites of action for the helicases: (i) at the level of transcription or translation to enhance or stabilize protein synthesis or (ii) in an association with DNA multisubunit protein complexes to alter gene expression. Here, we have described all the known plant helicases, which play a role in stress responses. The exploitation of abiotic stress-responsive helicase genes of new pathways of RNA and DNA unwinding will be helpful for engineering stress-tolerant crop plants.

19.1
Introduction

Stress is fundamentally a mechanical concept, defined by engineers and physical scientists as a force per unit area applied to an object. It is difficult to define stress so accurately in a biological sense. The most useful definition of biological stress is an "adverse force or influence that tends to inhibit normal system from functioning" [1].

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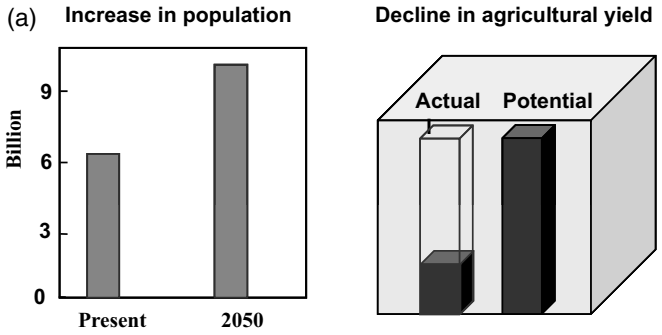
World population is increasing continuously and in 2050 it may reach more than 9 billion (http://www.unfpa.org/swp/200/); on the other hand, the crops' productivity is decreasing because of many negative factors including stresses (Figure 19.1a). Also, the demand for oilseeds, wheat, rice, pulses, and so on is going to much increase in near future (Table 19.1). Because of these factors, in future there may be danger to food security; therefore, it is important to develop stress-tolerant crops. Plants being immobile in nature have to bear a wide range of environmental stresses. They can respond to stress in several ways and have evolved mechanisms by which to increase their tolerance of these stresses through both physical adaptation and interactive molecular and cellular changes that begin after onset of stress [2]. Stresses can be broadly classified into two classes, namely, abiotic and biotic (Figure 19.1b). Low temperature, drought, and high salinity are common abiotic stress conditions.



(b)

Abiotic
1. Heat
2. Cold
3. Drought
4. Salinity
5. Wind
6. Waterlogging
7. Ozone
8. Heavy metals
9. Wounding
10. Nutrient loss
11. Excess light
12. Anoxia/Hypoxia
13. Genotoxic

Biotic (imposed by other organisms)
1. Pathogens (B,F,V)
2. Herbivores
3. Weeds
4. Insects
6. Nematodes
7. Mycoplasma

It is necessary to obtain stress-tolerant crops to cope with the upcoming problem of food security

Figure 19.1 (a) Population of the world is increasing, while the crop production is decreasing due to the negative impact of different stresses. (b) Different types of abiotic and biotic stresses and their negative effect leading to declining agricultural production. To cope up with the impending danger to food security, it is important to develop stress-tolerant crops.

Table 19.1 Target productivity requirement to meet the demand in 2020.

Food items	2009 Production (mt)	Demand 2020	Increase required
Oilseeds	30	8.5	243%
Pulses	35	~15	140%
Wheat	110	80	38%
Rice	130	~100	30%
Total cereals	285	220	30%

Source: FAO, 2009.

Biotic stresses include diseases caused by various pathogens. Overall, the environmental stresses adversely affect plant growth and productivity.

Abiotic stress in plants induces changes in the expression of many important genes, which affect the plant growth and development. Plants are continuously exposed to a plethora of different signals, which prevent them from reaching their full genetic potential. All these stress conditions adversely affect plant growth and limit agricultural production worldwide. To increase their tolerance, plants have developed mechanisms that involve both physical adaptation and interactive molecular and cellular changes. The responses to abiotic stresses are multigenic and the molecular mechanisms underlying these are not very well understood. The extracellular stress signal is first perceived by the membrane receptors and it then activates a large and complex signaling cascade intracellularly, including the generation of secondary signal molecules. The signal cascade results in the expression of multiple stress-responsive genes, the products of which can provide the stress tolerance directly or indirectly. Overall, the stress response could be a coordinated action of many genes, which may crosstalk with each other. Because the abiotic stresses affect the cellular gene expression machinery, it is possible that molecules involved in nucleic acid metabolism including helicase are likely to be the target. DNA helicases are motor proteins that catalyze the unwinding of duplex DNA in an ATP-dependent manner and thereby play an important role in most of the basic genetic processes including replication, repair, recombination, transcription, and translation [3–6] (Figure 19.2a). Usually, they need single-stranded (ss) DNA or ss/dsDNA junction as loading zone and translocate on DNA either in the 3′–5′ or in the 5′–3′ direction [3, 4].

All the helicases are also associated with intrinsic DNA-dependent ATPase activity, which provides energy for the helicase action [7, 8]. RNA helicases catalyze the ATP-dependent unwinding of local RNA secondary structures and play a broader role in remodeling RNA structures [9–12]. Many helicases share a core region (~200–700 amino acids) of highly conserved nine sequence motifs (designated Q, I, Ia, Ib, II, III, IV, V, and VI) and belong to the rapidly growing DEAD-box or DEAH-box protein family, which is conserved from bacteria to humans [6, 9–12] (Figure 19.2b). These conserved motifs are involved in different activities such as ATP-binding and hydrolysis, Mg^{2+} binding, DNA or RNA binding, unwinding, and so on (Figure 19.2b). Multiple DNA helicases are present in single cell in each system because of different structural requirement of the substrate at various stages of DNA

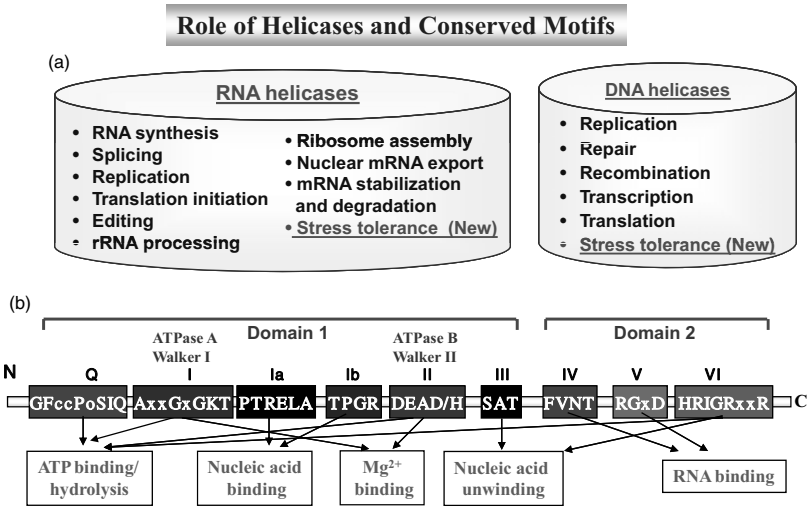


Figure 19.2 (a) Role of RNA and DNA helicases in different processes of nucleic acid metabolism. A new function in stress tolerance is also mentioned. (b) Helicase-conserved motif structure of DEAD and DEAH helicase family of proteins. Helicases are characterized by the presence of an Asp-Glu-Ala-Asp (DEAD) or an Asp-Glu-Ala-His (DEAH) motif (shown as motif II here). Both families of helicases contain amino-terminal (N) and carboxy-terminal domains (domain 1 and domain 2, respectively) and nine characteristic motifs (Q, I, Ia, Ib, and II–VI) within these. The known or proposed functions of each motif are shown.

transaction [3, 4, 13]. For example, at least 14 different DNA helicases have been reported from *Escherichia coli*, 6 from bacteriophages, 12 from viruses, 15 from yeast, 11 from calf thymus, and 34 from humans [5]. However, the exact biological roles of only few DNA helicases have been defined [3, 4, 6]. Still very little is known about DNA helicases from plant system. Till to date only 11 biochemically active DNA helicases have been reported in the purified form from plants [5, 14]. In plants, the exact role of plant DEAD-box helicases has not been defined properly. Recently, there have been some indications of a new role of helicases in stress-regulated processes.

19.2 Stress-Regulated Helicases

Many important crops and fruits that originated from the tropics or subtropics, such as rice, maize, tomato, banana, and orange, are injured or killed by exposure to low, nonfreezing temperature in the range of 0–12 °C [15, 16]. Low temperature is an important environmental factor that greatly influences the growth, development, survival, and distribution of plants [17]. Plant response to chilling are multigenic and the molecular mechanism of chilling sensitivity or resistance is not well understood [18]. Low temperature induces the expression of a diverse array of genes [19]. The product of these genes helps plants to adapt to subsequent freezing stress.

DEAD/H-box helicase genes were reported to induce under chilling and freezing stress [20, 21]. Although multiple DNA helicases have also been isolated from plants [5, 14], the molecular and biochemical characterization of the plant stress-induced DNA helicase(s) has not been achieved. Though the involvement of RNA helicase genes in response to stress has been reported from nonplant systems [22–24], the role of DNA helicases in stress has not been well studied. Analysis of genes whose expression is induced under stress condition is important to understand the mechanism of abiotic stress tolerance and possibly to use it for breeding stress-tolerant plants. However, the functions of DNA/RNA helicases are poorly understood in plants [25, 26]. Among all the sequenced genomes including those of humans, fly, worm, and yeast, *Arabidopsis* has the largest number of DEAD/H-box helicase genes [26–28]. There are 94 helicases reported from *Arabidopsis* (TAIR) that are regulated with stress. The Affymetrix 22 K ATH1 oligonucleotide expression data were obtained from the Genevestigator Response Viewer (https://www.genevestigator.com) [29] available as an external link in TAIR database.

19.3
Expression Profiling of *Arabidopsis* Helicase Genes under Abiotic Stress

The log2 fold expression values for 113 genes in various stresses such as anoxia, cold (3 independent replicates), drought, genotoxic, heat (2 independent replicates), hypoxia, osmotic, oxidative, salt, and wounding were imported into the Genesis software. The hierarchical clustering of 113 different transcriptomes revealed expression patterns for helicase genes under 10 different stress conditions. A dendrogram was constructed after integrating together the similar expression of genes into rows to form a cluster. The heat map resulting from the clustering analysis showed high expression of large set of helicases under anoxia, cold, and heat stresses. The expression analysis revealed overexpression of SDE3, RH55, chromatin remodeling 31, three genes for helicase domain-containing proteins, RH18 and RH11, in drought stress; RecQl3, helicase-related, CHR31 and MCM8 in genotoxic stress; MEE29, RH42, helicase domain-containing protein, SNF2, RH55, and MER3 in hypoxia. In osmotic stress, MEE29, RH55, CHR31, and RH45 showed increased expression, while in oxidative stress SDE3, helicase domain-containing protein, RH28, RNA helicase DRH1, and RH37 were overexpressed. The genes that showed high expression in salt stress were MEE29, SNF2 domain-containing protein, RH55, CHR31, CHR9, EDA16, RH30, RH40, and RNA helicase DRH1. In wounding stress, SNF2, CHR42, MER3, and PIF1 showed increased expression levels [28].

Since RNA molecules are more prone to forming stable nonfunctional secondary structures, their proper functioning requires RNA chaperones [28]. DEAD/H-box RNA helicases are the best candidates for RNA chaperones because these proteins can use energy derived from ATP hydrolysis to actively disrupt misfolded RNA structures so that correct folding can occur [28].

Kujat and Owttrim [30] reported that in photosynthetic organisms light-driven shift in redox potential acted as a sensor that initiates alteration in gene expression at

the level of both transcription and translation. This study provides evidence that the expression of a cyanobacterial RNA helicase gene *crhR* is controlled at the level of transcription and RNA stability by a complex series of mechanisms that are redox regulated. RNA helicase would not be directly involved in photosynthetic reaction *per se* to coordinate regulation of crhR expression implying that modulation of RNA secondary structure is required to elicit electron flow. CrhR-induced RNA unwinding activity could remove secondary structures that inhibit efficient translation of mRNAs whose products are required under these conditions. Whether crhR has specific RNA targets such as redox-induced mRNA, enhancing translation or assembly of ribonucleoprotein complex, or RNA turnover, remains to be explored [30].

19.3.1
Arabidopsis FL25A4 Helicase

Using biotinylated CAP trapper method, full-length cDNA libraries from *Arabidopsis* plants grown under different conditions such as drought treated, cold treated, or unstressed were constructed. By cDNA microarray analysis of 1300 *Arabidopsis* genes, Seki *et al*. [31] reported a DEAD-box helicase gene (accession number AB050574) as a cold stress-inducible gene suggesting a new role of helicases in stress signaling [25], but it has not been characterized further.

19.3.2
Arabidopsis LOS4 Helicase (AtRH38)

During a genetic screening for *Arabidopsis* mutants with deregulated expression of the *RD29A-LUC* reporter gene, a mutant named *los4-1* was isolated, which showed a reduced *RD29A-LUC* expression in response to cold, but not ABA or high salt. Northern blot analysis indicated that the mutation also decreases expression of endogenous RD29A and other COR/RD genes under cold stress. The CBF gene shows reduced or delayed cold induction in *los4-1* mutant plants. Unexpectedly, *los4-1* mutant plants are very sensitive to chilling temperature in dark. The constitutive expression of the CBF-3 gene reverses the chilling sensitivity of *los4-1* mutant plants. LOS4 gene was isolated by map-based cloning and found to encode for a DEAD-box RNA helicase protein (AtRH38) that is localized both in cytoplasm and in nucleus [21]. A novel *Arabidopsis* mutant (cryophyte) was isolated as having an enhanced cold induction of CBF2 and its downstream genes. Compared to wild type, mutant plants flower earlier and are smaller in size. The gene in this mutant was found to be a DEAD-box RNA helicase identical to LOS4 (low expression of osmotically responsive gene). Cryophyte was given the name *los4-2*. It has an RNA-dependent ATPase activity, and *los4-2* mutants are defective in mRNA export (Table 19.2). Consistent with its role in mRNA export, the LOS4 protein appears highly enriched at the nuclear rim. The *los4-2* and *los4-1* mutation affect cold response but in an opposite way. The *los4-1* plants appear to be sensitive to chilling stress, while *los4-2* show chilling resistance compared to the wild type. The *los4-2* mutant disrupts RNA export at warm

Table 19.2 Stress upregulated helicases from plants.

S. No.	Organism	Type of stress	Gene	Possible role	References
1.	*A. thaliana*	Low temperature (4 °C)	FL25A4	Suggested a new role for helicases in stress signaling	[31]
2.	*A. thaliana*	Low temperature (22–4 °C)	los-4-1, los-4-2	Involved in mRNA export	[21, 32]
3.	*Hordeum vulgare*	Salt and cold stress	HVD1	Regulates transcript(s) concerned with salt tolerance, or important metabolism such as photosynthesis, in chloroplast	[34]
4.	*Pisum sativum*	Salt stress	PDH45	Possible role in translation or regulating DNA/RNA metabolism under stress conditions	[35, 36]
5.	*P. sativum*	Salt stress and cold stress	PDH47	Efficient translation under stress condition or regulating the DNA/RNA metabolism	[37]
6.	*A. thaliana*	Salt, osmotic, and heat	STRS1 and STRS2	Mutations in either gene cause increased tolerance to salt, osmotic, and heat stresses, suggesting that the helicases suppress responses to abiotic stress	[38]
7.	*Apocynum venetum*	Salt and cold stress	AvDH1	ATP-dependent DNA helicase and ATP-independent RNA unwinding activities	[39]
8.	*M. sativa*	Mannitol, NaCl, methyl viologen, and abscisic acid	MH1	The ectopic expression of *MH1* in *Arabidopsis* improved seed germination and plant growth under drought, salt, and oxidative stress	[40]
9.	*A. thaliana*	Cold	AtRH9 and AtRH25	AtRH25, but not AtRH9, enhanced freezing tolerance in *Arabidopsis* plants	[41]
10.	*Glycine max* (soybean)	Low temperature and high salinity	GmRH	GmRH plays an important role in RNA processing during low-temperature and high-salinity stresses in plants	[43]
11.	*P. sativum*	High salinity and cold	MCM6	MCM6 single subunit from pea functions as DNA helicase and its overexpression in tobacco plant promotes salinity stress tolerance without affecting yield	[44, 45]

and high temperature but not at low temperature, while *los4-1* impairs mRNA export at warm and low temperature. So, *los4-1* appears to be a heat-sensitive allele that may even enhance mRNA export at low temperature, whereas *los4-1* appears to be a constitutive allele that affects mRNA export in both low and warm temperatures. Analysis of mRNA export-defective mutant cryophyte/*los4-2* has provided an uncommon opportunity to understand the contribution of mRNA export to higher plant development and stress response [32]. Overall, LOS4 helicase confers freezing tolerance by regulating mRNA export from the nucleus to the cytoplasm under cold stress conditions [21, 32, 33]. LOS4 helicase is also involved in many physiological processes such as germination (ABA hypersensitivity of los4-2) and plant development (los4 mutant flowers earlier), in addition to its role in low-temperature responses [32].

19.3.3
Sorghum HVD1 Helicase

In sorghum [34], a salt-responsive transcript HVD1 (*Hordeum vulgare* DEAD-box protein), encoding a putative ATP-dependent DEAD-box RNA helicase, was reported (Table 19.2). The transcript accumulation was induced under salt stress, cold stress, and ABA treatment. In addition to the conserved helicase domain, the encoded protein contained five repeats of RGG known as RNA recognition motif, at its hydrophilic C terminus. The transcript also dramatically increased during recovery from salt stress. The protein was found to localize in chloroplast by immunogold labeling. Thus, it was anticipated that HVD1 protein regulates the function of transcript(s) concerned with salt tolerance or important metabolism such as photosynthesis, in chloroplast. cDNA is essential for functional analysis of plant genes.

19.3.4
Pea DNA Helicase 45

It exhibits striking homology to eukaryotic translation initiation factor 4A (eIF4A) and contains ATP-dependent DNA and RNA helicase activity and DNA-dependent ATPase activity [35]. It is also reported that the pea DNA helicase 45 (*PDH45*) mRNA is upregulated in pea seedling in response to high salt (200 mM of NaCl), and when this gene was transferred to tobacco it provided the salinity stress tolerance [36]. This response was specific to Na^+ ion stress because treatment with Li^+ did not induce the transcript. This study was the first direct evidence of the possible role of a helicase in promoting the salinity stress tolerance in plants. The *PDH45* transcript was also upregulated in response to other abiotic stresses (dehydration, wounding, and low temperature), which suggested that the transcript increase could be due to water stress resulting from salinity- and mannitol-induced desiccation [36]. The induction of *PDH45* transcript was observed to be induced by the phytohormone, ABA, which suggested that the stress effect may take place through ABA-mediated pathways. The exact mechanism of PDH45-mediated tolerance to salinity stress is not understood. This protein may act at translational level or may associate with DNA multisubunit protein complex to alter gene expression [36].

19.3.5
Pea DNA Helicase 47 (PDH47)

The pea DNA helicase 47 (PDH47) also belongs to DEAD-box protein family and shows 93% homology to tobacco eIF4A. The purified recombinant protein (47 kDa) was reported to contain ATP-dependent DNA helicase and DNA-dependent ATPase activities. These activities are upregulated after phosphorylation of PDH47 at Ser and Thr residues with protein kinase C. Using Western blot analysis and *in vivo* immunostaining followed by confocal microscopy, PDH47 is localized in the nucleus and cytosol. The level of transcript of *PDH47* is more in shoot than in root. The transcript was induced in both shoot and root under cold (4 °C) and salinity (300 mM of NaCl) stress, but there was no change in response to drought stress. It is a unique bipolar helicase that contains both the 3′–5′ and 5′–3′ directional helicase activities. The anti-PDH47 antibodies immunodeplete the activities of PDH47 and inhibit *in vitro* translation of protein. Furthermore, the PDH47 protein showed induction of protein synthesis [37].

19.3.6
Arabidopsis STRS1 and STRS2

Two DEAD-box RNA helicases from *Arabidopsis* were reported to be downregulated by multiple abiotic stresses. However, the mutations in their coded genes resulted in increased tolerance to salt, osmotic, and heat stresses (Table 19.2). This suggested that these helicases suppress responses to abiotic stress. The genes were, therefore, named stress response suppressor 1 (STRS1; At1g31970) and STRS2 (At5g08620) [38]. These mutants showed greater tolerance than wild type to multiple abiotic stresses and also showed more highly induced expression of genes encoding stress-responsive transcription factors and their downstream target genes. The ABA is observed to reduce the expression of the STRS genes, but the STRSs were reported to be regulated by both ABA-dependent and -independent stress signaling networks. Overall, this study indicated the importance of RNA metabolism in the control of stress-responsive gene expression.

19.3.7
Dogbane AvDH1 Helicase

The AvDH1 helicase is a salt-responsive gene isolated from the halophyte dogbane (*Apocynum venetum*). It also contained the nine conserved helicase motifs of the DEAD-box protein family. The purified recombinant protein contains ATP-dependent DNA and RNA helicase activities and DNA- or RNA-dependent ATPase activities. The *AvDH1* gene was reported to be present as a single copy in the dogbane genome. This gene was found to be upregulated in response to NaCl and not in drought and abscisic acid. The AvDH1 transcript was also induced by cold stress, but its accumulation was first increased then decreased with time [39].

19.3.8
Alfalfa MH1 Helicase

The cDNA of this helicase was cloned from *Medicago sativa* (alfalfa) and was found to be homologous to PDH45, and was named M. sativa helicase 1 (MH1). The *MH1* gene was found to be expressed in roots, stems, and leaves, and was upregulated in response to mannitol (drought), NaCl, or H_2O_2 treatments (Table 19.2). The expression of *MH1* in *Arabidopsis thaliana* conferred tolerance to drought and salinity to the transgenic plants. The enhanced stress tolerance in MH1-expressing *Arabidopsis* was observed to be correlated with an increase in superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities and proline content. The findings suggested that MH1may function in abiotic stress by elevating the capacities for reactive oxygen species (ROS) scavenging and osmotic adjustment [40].

19.3.9
Arabidopsis AtRH9 and AtRH25

Two *Arabidopsis* helicases, AtRH9 (At3g22310) and AtRH25 (At5g08620), were selected by Kim *et al.* [41] to study the basis of the observation that these two DEAD-box RNA helicases were among the genes highly upregulated in the transcriptome of *Arabidopsis* plants subjected to cold stress [42]. Both these helicases were found to be upregulated in response to cold stress, whereas their transcript levels were downregulated by salt or drought stress (Table 19.2). Phenotypic analysis of the overexpression of AtRH9 or AtRH25 transgenic plants showed the retarded seed germination of *Arabidopsis* plants under salt stress conditions. AtRH25, but not AtRH9, was also reported to enhance freezing tolerance in *Arabidopsis* plants [41].

19.3.10
Soybean GmRH

A novel RNA helicase GmRH has been isolated from soybean and characterized by Chung *et al.* [43]. This helicase was shown to contain a bipartite lysine-rich nuclear localization signal (NLS) to ward the N-terminal variable region of GmRH. The soybean genome was reported to contain two copies of *GmRH* gene. The gene was reported to be upregulated in response to low-temperature or high-salinity stress, but not in response to abscisic acid or drought stress. The GmRH recombinant protein contained dsRNA unwinding activity independent of ATP *in vitro*. The authors proposed that GmRH might play an important role in RNA processing during low-temperature and high-salinity stresses in plants (Table 19.2).

19.3.11
Pea MCM6 Single-Subunit DNA Helicase

The eukaryotic prereplicative complex (Pre-RC), including heterohexameric mini-chromosome maintenance (MCM2–7) proteins, ensures that the DNA in genome is

replicated only once per cell division cycle. The MCM complex provides DNA unwinding function during the DNA replication. However, the unwinding function in the single subunit of MCM and its role in abiotic stress tolerance were not reported so far in any systems. Recently, we have shown the first direct evidence of the identification of a DNA unwinding activity in a single-subunit (MCM6) of the MCM complex of pea [44]. The pea MCM6 single subunit is also reported to form a homohexamer that actually functions as a DNA helicase (Table 19.2). The DNA helicase activity is in 3′–5′ direction and the activity is found to be stimulated by replication fork-like structure of the substrate [44]. Since MCM proteins play an essential role in cell division and most likely are affected during stress conditions, their overexpression in plants may help in stress tolerance. Furthermore, we have tested its role in salinity stress tolerance. Recently, we have reported that (a) the MCM6 transcript is upregulated in pea plant in response to high-salinity and cold stress and not with ABA, drought, and heat stress (Table 19.2); (b) MCM6 overexpression driven by a constitutive cauliflower mosaic virus-35S promoter in tobacco plants confers salinity tolerance. The T_1 transgenic plants were able to grow to maturity and set normal viable seeds under continuous salinity stress, without yield penalty. It was observed that in salt-grown T_1 transgenic plants, the Na^+ ions is mostly accumulated in mature leaves and not in seeds of T_1 transgenic lines compared to the wild-type (WT) plants. T_1 transgenic plants exhibited better growth status under salinity stress conditions in comparison to WT plants. Furthermore, the T_1 transgenic plants maintained significantly higher levels of leaf chlorophyll content, net photosynthetic rate, and therefore higher dry matter accumulation and yield with 200 mM of NaCl compared to WT plants. Tolerance index data showed better salt tolerance potential of T_1 transgenic plants in comparison to WT. These findings provide first direct evidence that overexpression of single-subunit MCM6 confers salinity stress tolerance without yield loss [45]. The possible mechanism of salinity tolerance is discussed. These findings suggest that DNA replication machinery can be exploited for promoting stress tolerance in crop plants.

19.4
Possible Mechanisms of Helicase Action During Stress

The exact mechanism of helicase-mediated tolerance of stress has not yet been understood. There could be two possible sites of action for the helicases: (i) at the level of transcription or translation to enhance or stabilize protein synthesis or (ii) in an association with DNA multisubunit protein complexes to alter gene expression. It is evident that mRNA and protein synthesis are very sensitive to stress, so factors involved in transcription and translation are potential targets of salt toxicity in plants. In bacteria, the toxic effect of Na^+ is mainly in translation rather than in RNA synthesis. The mechanisms of translation initiation are conserved among eukaryotes and the regulation of translation occurs at the step of initiation. The RNA helicase activity of DEAD-box proteins could facilitate transcription by altering the structure of nascent RNA, a process that can stimulate reinitiation and/or elongation.

The initiation step of translation is impaired after cold shock and the reactivation of translation machinery might represent a bottleneck during cold adaptation. RNA helicases not only remove RNA secondary structure but also protect mRNA from degradation in particular under condition where transcription and translation are uncoupled as a result of inefficient translation initiation [46].

There are also temperature-dependent changes in the cellular ribosome profile. Upon cold shock, the number of polysomes existing at optimal temperature decreases in favor of increasing amounts of monosomes, 70 S particles, and free ribosomal 30 S and 50 S subunits. This effect has been suggested to result from a cold-induced block in translation initiation. As a consequence, additional mRNA structuring may occur that would further complicate protein biosynthesis at low temperature [47]. There is a temperature-dependent alteration of mRNA structures affecting ribosomal protein synthesis. The mRNA secondary structures at the 5′ untranslated region (5′UTR) can mask the ribosomal binding site, and at 3′UTR can mask the stop codon, which finally

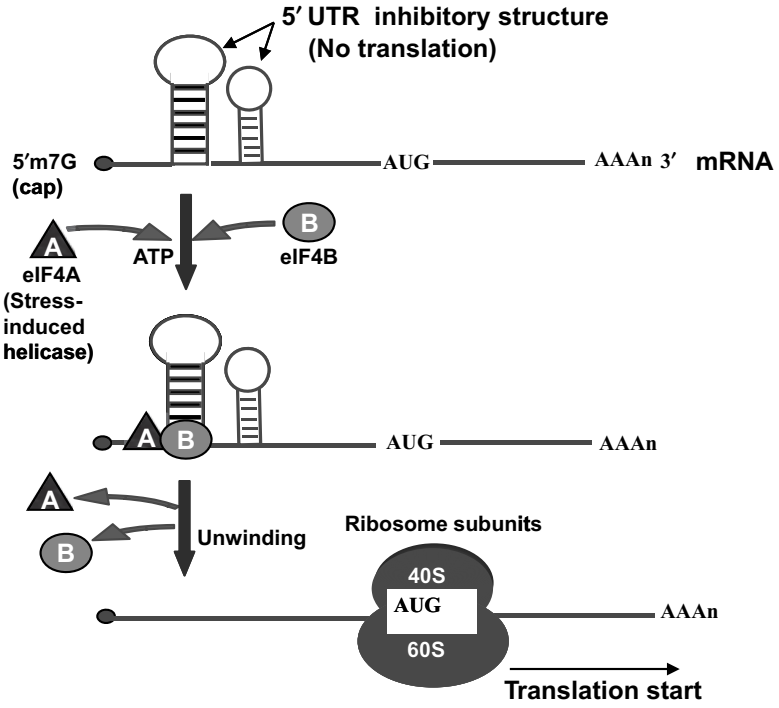


Figure 19.3 Hypothetical model for the possible mechanism of stress tolerance by a helicase. The eIF4A is a prototypic member of the DEAD-box RNA helicase family. Stresses may enhance formation of the inhibitory secondary structure at the 5′UTR of mRNAs of many essential genes. The eIF4A is responsible for removal of the secondary structure of the mRNA. eIF4A, along with eIF4B, binds to 5′UTR and unwinds the inhibitory secondary structure in an ATP-dependent manner. This facilitates the binding of ribosome. After this, ribosome scans for the start codon (AUG) and protein synthesis begins normally, which was inhibited due to negative impact of the stress.

leads to impairment of protein translation initiation. The stress-induced helicases may resolve these inhibitory structures during stress adaptation.

The possible mechanism of helicase action during stress is depicted in Figure 19.3. In response to stress, the extra secondary structures could be formed in the 50-untranslated region in mRNA of many essential genes, which could be inhibitory for translation. These inhibitory secondary structures need to resolve in order to have active translation, as otherwise these RNAs will act as nonfunctional RNAs where protein synthesis cannot proceed. The stress-induced RNA helicase(s) recognized these nonfunctional RNAs and unwound to resolve the secondary structures, which permit the translation initiation to proceed (Figure 19.3). Overall, these stress-induced helicases help in recovering the functions of the genes for stress adaptation, which were stopped previously because of the negative impact of the stress.

The involvement of DEAD-box helicases in various metabolic processes in plant cells might have general implications. In plants, the role of these helicases in stress responses is just beginning to be understood. The overexpression of stress-induced DEAD-box helicase(s) can provide an example of the exploitation of DNA/RNA metabolism pathways for engineering stress-tolerant crop plants. Overall, DEAD-box helicases are conserved and have emerged as new molecules to understand stress signaling in plants. A few studies of stress-induced DNA and RNA helicases suggested that salinity stress affects the stability of nucleic acid base pairing. Therefore, the exploitation of salinity stress-responsive genes of new pathways, including DNA/RNA metabolism, will be useful in elucidating the less-known stress signaling networks and will also be helpful for engineering salinity-tolerant crop plants.

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References

1 Jones, H.G. and Jones, M.B. (1989) Introduction: some terminology and common mechanisms, in *Plant Under Stress* (eds H.G. Jones, T.J. Flowers, and M.B. Jones), Cambridge University Press, Cambridge, pp. 1–10.

2 Knight, H. and Knight, M.R. (2001) Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci.*, **6**, 262–267.

3 Matson, S.W., Bean, D., and George, J.W. (1994) DNA helicases; enzymes with essential roles in all aspects of DNA metabolism. *BioEssays*, **16**, 13–21.

4 Lohman, T.M. and Bjornson, K.P. (1996) Mechanism of helicase-catalyzed DNA unwinding. *Ann. Rev. Biochem.*, **65**, 169–214.

5 Tuteja, N. and Tuteja, R. (2004) Prokaryotic and eukaryotic DNA helicases: essential molecular motor proteins for cellular machinery. *Eur. J. Biochem.*, **271**, 1835–1848.

6 Tuteja, N. and Tuteja, R. (2004) Unraveling DNA helicases: motif, structure, mechanism and function. *Eur. J. Biochem.*, **271**, 1849–1863.

7 Hall, M.C. and Matson, S.W. (1999) Helicase motifs: the engine that powers DNA unwinding. *Mol. Microbiol.*, **34**, 867–877.

8 Rocak, S. and Linder, P. (2004) DEAD-box proteins: the driving forces behind RNA metabolism. *Nat. Rev. Mol. Cell Biol.*, **5**, 232–241.

9 Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P., and Blinov, V.M. (1989) Two related superfamilies of putative helicases involved in replication, recombination, repair and expression DNA and RNA genomes. *Nucleic Acids Res.*, **17**, 4713–4730.

10 Linder, P., Lasko, P.F., Ashburner, M., Leroy, P., Nielsen, P.J., Nishi, K., Schnier, J., and Slonimski, P.P. (1989) Birth of the D-E-A-D box. *Nature*, **337**, 121–122.

11 Pause, A. and Sonenberg, N. (1992) Mutational analysis of a DEAD-box RNA helicase; the mammalian translation initiation factor eIF-4A. *EMBO J.*, **11**, 2643–2654.

12 Luking, A., Stahl, U., and Schmidt, U. (1998) Protein family of RNA helicases. *Crit. Rev. Biochem. Mol. Biol.*, **33**, 259–296.

13 Tuteja, N. and Tuteja, R. (1996) DNA helicases: the long unwinding road. *Nat. Genet.*, **13**, 11–12.

14 Tuteja, N. (2003) Plant DNA helicases: the long unwinding road. *J. Exp. Bot.*, **54**, 2201–2214.

15 Graham, D. and Patterson, B.D. (1982) Responses of plants to low, non-freezing temperatures: proteins, metabolisms, and acclimation. *Annu. Rev. Plant Physiol.*, **33**, 347–372.

16 Lyons, J.M. (1973) Chilling injury in plants. *Ann. Rev. Plant Physiol.*, **24**, 445–466.

17 Levitt, J. (1980) *Responses of Plants to Environmental Stresses: Chilling, Freezing, and High Temperature Stress*, 2nd edn, Academic Press, New York.

18 Tokuhisa, J. and Browse, J. (1999) Genetic engineering of plant chilling tolerance. *Genet. Eng. (NY)*, **21**, 79–93.

19 Thomashow, M.F. (2001) So what's new in the field of plant cold acclimation? Lots!. *Plant. Physiol.*, **125**, 89–93.

20 Chamot, D., Magee, W.C., Yu, E., and Owttrim, G.W. (1999) A cold shock-induced cyanobacterial RNA helicase. *J. Bacteriol.*, **181**, 1728–1732.

21 Gong, Z., Lee, H., Xiong, L., Jagendorf, A., Stevenson, B., and Zhu, J.K. (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc. Natl. Acad. Sci. USA*, **99**, 11507–11512.

22 Briolat, V. and Reysset, G. (2002) Identification of the *Clostridium perfringens* genes involved in the adaptive response to oxidative stress. *J. Bacteriol.*, **184**, 2333–2343.

23 Liu, H.Y., Nefsky, B.S., and Walworth, N.C. (2002) The Ded1 DEAD-box helicase interacts with Chk1 and Cdc2. *J. Biol. Chem.*, **277**, 2637–2643.

24 Westermarck, J., Weiss, C., Saffrich, R., Kast, J., Musti, A.M., Wessely, M., Ansorge, W., Seraphin, B., Wilm, M., Valdez, B.C., and Bohmann, D. (2002) The DEXD/H-box RNA helicase RHII/Gu is a co-factor for c-Jun-activated transcription. *EMBO J.*, **21**, 451–460.

25 Lorkovic, Z.J., Herrmann, R.G., and Oelmuller, R. (1997) PRH75, a new nucleus-localized member of the DEAD-box protein family from higher plants. *Mol. Cell. Biol.*, **17**, 2257–2265.

26 Aubourg, S., Kreis, M., and Lecharny, A. (1999) The DEAD box RNA helicase family in *Arabidopsis thaliana*. *Nucleic Acids Res.*, **27**, 628–636.

27 Boudet, N., Aubourg, S., Toffano-Nioche, C., Kreis, M., and Lecharny, A. (2001) Evolution of intron/exon structure of DEAD helicase family genes in *Arabidopsis*, *Caenorhabditis*, and *Drosophila*. *Genome Res.*, **11**, 2101–2114.

28 Umate, P., Tuteja, R., and Tuteja, N. (2010) Genome wide analysis of helicase gene family from rice and *Arabidopsis*: a comparison with yeast and human. *Plant Mol. Biol.*, **73**, 449–465.

29 Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., and Zimmermann, P. (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics*, 420747.

30 Kujat, S.L. and Owttrim, G.W. (2000) Redox-regulated RNA helicase expression. *Plant Physiol.*, **124**, 703–714.

31 Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. (2001) Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell*, **13**, 61–72.

32 Gong, Z., Dong, C.H., Lee, H., Zhu, J., Xiong, L., Gong, D., Stevenson, B., and Zhu, J.K. (2005) A DEAD Box RNA helicase is essential for mRNA export and important for development and stress responses in *Arabidopsis*. *Plant Cell*, **17**, 256–267.

33 Zhu, J., Dong, C.-H., and Zhu, J.-K. (2007) Interplay between cold responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr. Opin. Plant Biol.*, **10**, 290–295.

34 Nakamura, T., Muramoto, Y., and Takabe, T. (2004) Structural and transcriptional characterization of a salt-responsive gene encoding putative ATP-dependent RNA helicase in barley. *Plant Sci.*, **167**, 63–70.

35 Pham, X.H., Reddy, M.K., Ehtesham, N.Z., Matta, B., and Tuteja, N. (2000) A DNA helicase from *Pisum sativum* is homologous to translation initiation factor and stimulates topoisomerase I activity. *Plant J.*, **24**, 219–229.

36 Sanan-Mishra, N., Pham, X.H., Sopory, S.K., and Tuteja, N. (2005) Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. *Proc. Natl. Acad. Sci. USA*, **102**, 509–514.

37 Vashisht, A., Pradhan, A., Tuteja, R., and Tuteja, N. (2005) Cold and salinity stress-induced pea bipolar pea DNA helicase 47 is involved in protein synthesis and stimulated by phosphorylation with protein kinase C. *Plant J.*, **44**, 76–87.

38 Kant, P., Kant, S., Gordon, M., Shaked, R., and Barak, S. (2007) STRESS RESPONSE SUPPRESSOR1 and STRESS RESPONSE SUPPRESSOR2: two DEAD-box RNA helicases that attenuate *Arabidopsis* responses to multiple abiotic stresses. *Plant Physiol.*, **145**, 814–830.

39 Liu, H.H., Liu, J., Fan, S.L., Song, M.Z., Han, X.L., Liu, F., and Shen, F.F. (2008) Molecular cloning and characterization of a salinity stress induced gene encoding DEAD-box helicase from the halophyte *Apocynum venetum*. *J. Exp. Bot.*, **59**, 633–644.

40 Luo, Y., Liu, Y.B., Dong, Y.X., Gao, X.Q., and Zhang, X.S. (2009) Expression of a putative alfalfa helicase increases tolerance to abiotic stress in *Arabidopsis* by enhancing the capacities for ROS scavenging and osmotic adjustment. *J. Plant Physiol.*, **166**, 385–394.

41 Kim, J.S., Kim, K.A., Oh, T.R., Park, C.M., and Kang, H. (2008) Functional characterization of DEAD-Box RNA helicases in *Arabidopsis thaliana* under abiotic stress conditions. *Plant Cell Physiol.*, **49**, 1563–1571.

42 Fowler, S. and Thomashow, M.F. (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell*, **14**, 1675–1690.

43 Chung, E., Cho, C.W., Yun, B.H., Choi, H.K., So, H.A., Lee, S.W., and Lee, J.H. (2009) Molecular cloning and characterization of the soybean DEAD-box RNA helicase gene induced by low temperature and high salinity stress. *Gene*, **443**, 91–99.

44 Tran, N.Q., Dang, H.Q., Tuteja, R., and Tuteja, N. (2010) A single subunit MCM6 from pea forms homohexamer and functions as DNA helicase. *Plant Mol. Biol.*, **74**, 327–336.

45 Dang, H.Q., Tran, N.Q., Gill, S.S., Tuteja, R., and Tuteja, N. (2011) A single subunit MCM6 from pea promotes salinity stress tolerance without affecting yield. *Plant Mol. Biol.* doi: 10.1007/s11103-011-9758-0

46 Iost, I. and Dreyfus, M. (1994) mRNAs can be stabilized by DEAD-box proteins. *Nature*, **372**, 193–196.

47 Weber, M.H. and Marahiel, M.A. (2003) Bacterial cold shock responses. *Sci. Prog.*, **86**, 9–75.

20
Transcription Factors: Improving Abiotic Stress Tolerance in Plants

Pil Joon Seo, Jae-Hoon Jung, and Chung-Mo Park

Perception of environmental cues and downstream cellular signaling schemes are critical for plant adaptation and survival under abiotic stress conditions. Gene transcription is a primary regulatory scheme that induces massive biological processes and traits in response to incoming signals. In this regard, transcription factors play a critical role in gene regulatory networks governing cellular and organismal responses to developmental signals, including those mediated by growth hormonal regulators, and environmental fluctuations, such as cold or low temperatures, high salinity, and drought. Numerous transcription factors and their target genes have been identified, and underlying molecular mechanisms have been explored in a variety of plant species, mostly in *Arabidopsis* and rice. Furthermore, it has been shown that there are extensive signaling crosstalks among different environmental signals. Therefore, understanding the roles of major transcription factors in stress adaptation responses and their signaling interactions is important for genetic engineering of crop plants to improve stress tolerance. In this chapter, transcriptional signaling cascades under various abiotic stress conditions, roles of transcription factors, and their regulatory schemes are discussed.

20.1
Introduction

Gene expression regulation is a fundamental step in modulating plant growth and developmental processes and environmental adaptation responses. Gene transcription is regulated by transcription factors, which perceive developmental and environmental cues. Therefore, understanding transcription factor activities and underlying molecular mechanisms provides information on molecular and physiological changes occurring in plant responses to various abiotic stress conditions [1, 2].

Gene transcriptional regulation is established by coordinate interactions between *cis*-acting elements of gene promoters and transcription factors. The *cis*-acting elements determine the type, affinity, and arrangement of transcription factors and

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associated regulatory proteins [3]. Transcription factors also have differential DNA binding affinities, transcriptional activation/repression activities, and capacities of protein–protein interaction, suggesting that elaborate combinatorial interactions between transcription factors and gene promoters allow precise control of gene transcription [3, 4].

Transcription factors are usually defined as sequence-specific DNA binding proteins, which are capable of activating or suppressing gene transcription [3]. Transcription factors have a modular structure, which consists of DNA binding domain (BD), protein–protein interaction domain, and transcriptional regulation domain [1]. DNA binding domain facilitates binding of transcription factors to specific DNA sequences. According to the structural features of DNA biding domains, transcription factors are grouped into families [5]. Approximately, 64 DNA binding domains have been defined in *Arabidopsis* transcription factors [4, 5]. Protein–protein interaction domains are responsible for interactions with both its own and diverse transcription factors and its isoforms, contributing to regulational diversity and specificity. Gene transcriptional regulation domains function in harmony with regulatory proteins that interact with RNA polymerase [1]. These domains determine the efficiency of assembly of basal transcriptional complex and binding to RNA polymerase II [1]. Their activities are resulted from acidic or hydrophobic residues residing in the domains [3].

Transcription factors are regulated by diverse environmental and developmental signals to activate/suppress target genes involved in stress adaptation and developmental optimization, and thus they are important and efficient signaling mediators between external/internal signals and plant responses [6, 7]. Signaling network is a dense web of numerous regulatory proteins and responsive genes. Therefore, it is not surprising that individual transcription factors belonging to the same family are differently regulated by incoming signals. In addition, those belonging to different transcription factor families often share same targets, indicating that complex networks and crosstalks are established by transcription factors [7, 8].

In this chapter, we will emphasize transcriptional regulation of abiotic stress responses. We will also cover general interactions between transcription factors and *cis*-elements that are involved in abiotic stress responses and roles of various transcription factors in establishing abiotic stress resistance with concrete examples. Furthermore, functional mechanisms underlying transcription factor activities are also discussed.

20.2 Abiotic Stress Responses

Drought and salt stresses, together with cold stress, greatly affect agronomic yield and productivity. Perception of environmental stresses and downstream signaling cascades to activate adaptive stress responses are key steps to acquire stress resistance [7, 9, 10]. Various abiotic stresses impose both general and specific effects

on plant growth and development. Molecular and genetic studies, in particular with model plants *Arabidopsis* and rice, have elucidated diverse mechanisms and signaling networks that are involved in stress signaling and responses.

Many transcriptional regulators have been identified by transcriptome analysis. It has been found that transcription factor genes constitute a significant proportion of stress-inducible genes, suggesting that gene transcriptional regulation of stress responses is a crucial mechanism for stress adaptation [8]. These transcriptional regulators include diverse transcription factor family members, such as basic helix–loop–helix (bHLH), basic leucine zipper (bZIP), APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (AP2/ERF), homeodomain, MYB, *N*AM/*A*TAF1,2/*C*UC2 (NAC), WRKY, and zinc finger transcription factors [7].

Transcription factors establish complicated signaling cascades in abiotic stress responses. These signaling cascades are classified on the basis of several criteria. A substantial proportion of the genes induced by osmotic stresses, such as salt, cold, and drought, are also regulated by abscisic acid (ABA) [7]. Accordingly, involvement of ABA is used as an important criterion in dividing stress signaling pathways. ABA dependence of stress response is investigated by ABA-deficient *aba* mutants and ABA-signaling mutants, such as *abi* (ABA-insensitive). A number of genes are completely independent of ABA, indicating that both ABA-dependent and ABA-independent pathways mediate stress adaptation responses [11]. It has been found that while more than half of the drought-inducible genes are also induced by high salt and/or ABA, only 10% of them are regulated by cold stress [11]. Responses to drought, high salt, and ABA are highly linked through extensive crosstalks, but cold stress responses are relatively separated from ABA signaling [11].

Signaling cascades governing stress responses can be understood by examining the interactions of transcription factors with *cis*-acting elements. Transcription factors regulate their target genes through direct binding to target gene promoters. In this regard, promoter analysis provides clues as to the interdependence and/or independence between different abiotic stress signaling pathways [6, 7]. Promoters of abiotic stress-responsive genes usually contain C-repeat/dehydration responsive element (CRT/DRE), ABA-responsive element (ABRE), and recognition sequences of MYC and MYB transcription factors [7]. The ABRE (PyACGTGGC) functions as a *cis*-acting element in many ABA-responsive genes [12]. High salt and drought stresses induce ABA accumulation, which activates osmotic stress responses. Therefore, most of high salt- and drought-inducible genes contain the ABRE in their promoters. ABA-dependent signaling usually activates bZIP transcription factors, including ABRE-binding factors/AREB-binding proteins (ABFs/AREBs), which induce stress-responsive genes by directly binding to the ABRE [12]. Interactions of MYB and MYC transcription factors with its recognition sequences also constitute ABA-dependent signaling [13].

While ABA-dependent stress-responsive genes are regulated through the ABRE, ABA-independent stress-responsive genes are regulated mainly via the DRE *cis*-acting element (TACCGACAT) [7, 14]. The C-repeat binding factor/DRE-binding protein (CBF/DREB) transcription factors belonging to the AP2/ERF family bind

specifically to the DRE *cis*-acting element to activate stress-responsive genes [6]. Compared to high salt and drought stresses, cold stress is relatively independent of ABA signaling pathways [11, 14]. Mutations harboring ABA-mediated *RESPONSIVE TO DESICCATION 29A* (*RD29A*) induction increase salt and drought tolerance but not cold, implying that cold stress responses are partially separated from ABA-mediated osmotic stress responses [7, 14]. ABA accumulation in response to cold stress is still controversial. Although ABA contributes to regulation of cold-responsive gene expression and freezing tolerance, its effects is not great as those of high salt and drought stresses. These observations may be due to the existence of DRE *cis*-acting element-mediated ABA-independent signaling pathway in cold stress responses [6] (Figure 20.1).

Interactions and convergence of signalings are largely mediated through *cis*-acting elements. The promoter of the *RD29A* gene contains four DRE-like sequences and one ABRE. As inferred from the presence of a series of distinct *cis*-acting elements in the gene promoter, the *RD29A* gene is induced by ABA, dehydration, and cold, supporting the view that the *cis*-acting elements existing in the gene promoter are one

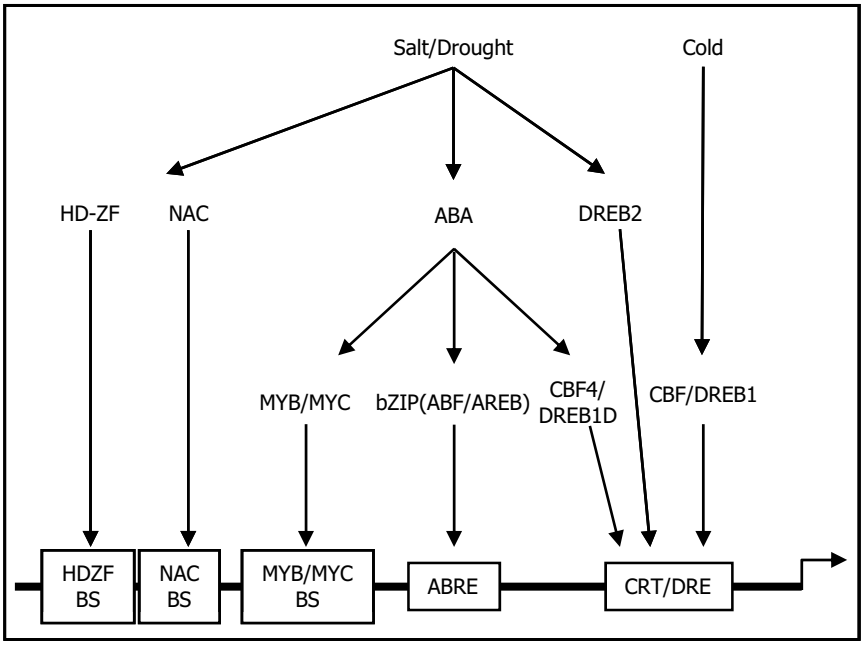

Figure 20.1 The *trans*-acting regulators and *cis*-acting elements involved in abiotic stress signaling. Specific interactions between stress-inducible transcription factors and *cis*-acting elements determine specificity and strength of downstream signaling. Note that multiple *trans*-acting regulators bind to a *cis*-acting element either cooperatively or competitively to fine-tune downstream signaling. This entails that there are extensive signaling crosstalks between different stress signalings.

of the major sites for signal convergence [7, 14]. Consistent with this view, the gene induction patterns in response to dehydration and cold stresses are maintained in the ABA biosynthetic and signaling mutants, indicating that the *RD29A* gene integrates both the ABA-dependent and the ABA-independent signals via the interaction of *cis*-acting elements [7, 14].

Interactions between *cis*-elements are also important for a proper regulation of stress-inducible genes. The DRE *cis*-acting element is sufficient for stress-responsive gene induction. While ABA is required for full induction of the DRE, it cannot directly activate the DRE [11]. It is suspected that interactions between the DRE and ABREs are important for full activation of target genes [11]. As described above, the *RD29A* gene promoter contains four DRE-like sequences and one ABRE. Although single ABRE cannot efficiently perceive ABA signals, the DRE–ABRE interactions seem to be necessary to mediate webs of ABA signalings [11].

Plants are frequently exposed to more than one stress under given growth conditions. Therefore, plants evolved complicated mechanisms to resist various environmental stresses through extensive crosstalks and feedbacks [6]. In other word, improvement of resistance to one stress can induce tolerant response to other stresses. We will further categorize transcription factors according to stress signaling involved. However, signaling crosstalks and interrelationship should be considered.

20.3
Transcription Factors in Stress Adaptation

20.3.1
Drought Stress

Several genes involved in ABA biosynthesis are induced by drought stress [7, 15]. The ABA biosynthetic genes encoding 9-*cis*-epoxycarotenoid dioxygenase (NCED) are transcriptionally regulated by drought stress, supporting the view that ABA is intimately linked to drought stress responses [16]. As a result, a significant proportion of drought inducible genes is also induced by ABA.

The bZIP transcription factors ABFs/AREBs are the best characterized transcription factors that function in drought stress response via ABA-dependent signaling [7, 12]. The ABF1, ABF2/AREB1, ABF3, ABF4/AREB2, and ABEB3 transcription factors require ABA to activate their own activity through ABA-dependent phosphorylation. Therefore, they exhibit reduced activity in the *aba* and *abi* mutants, but increased activity in the ABA-hypersensitive *era1* mutant [17]. Although loss-of-function mutants do not have any obvious phenotypes, constitutive expression of either *ABF3* or *ABF4*/*AREB2* leads to ABA hypersensitivity, reduced transpiration, stomatal closure, enhanced drought resistance, and altered expression of ABRE-containing genes, such as *RD29B*, *ABI1*, and *ABI2* [12].

There are additional pathways to regulate drought stress responses in an ABA-dependent manner. Although the *RD22* gene is induced by ABA and drought stress,

the ABRE does not exist in the *RD22* gene promoter. Instead, MYC and MYB transcription factors cooperatively regulate drought stress responses [13]. The AtMYC2 (RD22BP1) and AtMYB2 transcription factors bind directly to the *cis*-acting element present in the *RD22* gene promoter [13].

Additional MYB transcription factors also serve as drought stress regulators in *Arabidopsis*. The MYB96 transcription factor coordinately regulates stomatal aperture, shoot growth, lateral root development, and expression of the *RD22* gene and a subset of *GH3* genes to optimize plant growth under drought conditions [18]. Accordingly, while transgenic plants overexpressing the *MYB96* gene exhibit enhanced drought tolerance, the *myb96-1* knockout mutant is susceptible to drought stress [18]. The MYB44 transcription factor also regulates stomatal aperture in response to ABA, drought, and high salinity. Transgenic plants overexpressing the *MYB44* gene exhibit enhanced tolerance to drought and high salt stresses, stomatal closure, and reduced water loss [19]. Microarray analysis have shown that a group of protein phosphatase 2C (PP2Cs) genes is negatively regulated by MYB44, resulting in constitutive activation of ABA signaling [19].

Two R2R3-type MYB transcription factors, MYB60 and MYB61, have been identified as stomatal movement regulator. The *MYB60* gene is expressed specifically in guard cells and repressed in response to drought stress. Consistent with this, loss-of-function mutants of the *MYB60* gene show reduced stomatal aperture and enhanced drought resistance [20]. The *MYB61* gene is also expressed specifically in guard cells and controls stomatal aperture according to light/dark responses, suggesting a specific role of the MYB transcription factors in regulation of stomatal function [21].

A WRKY transcription factor is also involved in ABA-mediated drought tolerance response. Loss-of-function mutants of *AtWRKY63* are less sensitive to ABA in stomatal closure. In addition, drought resistance in the mutants is reduced [22]. AtWRKY63 regulates the *ABF2*/*AREB1* gene transcription by binding to the W-box existing in the gene promoter and affects expression of the *RD29A* and *COR47* genes [22].

Nuclear Factor (NF) is a transcription factor with high DNA binding affinity to the CCAAT box [23]. A subunit of NF-Y (NF-YA) confers the specific binding capacity to DNA by forming a trimeric complex (NF-YA, NF-YB, and NF-YC) [24]. Among the 10 members of NF-YAs, the *NFYA5* gene is induced by drought stress via an ABA-dependent signaling [23]. In addition, miR169 regulates the *NFYA5* transcript. The miR169 expression is downregulated by drought stress via ABA-dependent pathways, upregulating the *NFYA5* gene. Accordingly, whereas transgenic plants overexpressing *NFYA5* exhibit enhanced drought tolerance, the *nfya5* mutant and miR169-overproducing transgenic plants show higher susceptibility to drought stress with elevated water loss [23]. Related transcription factors NFYB1 and LEC1, members of the NFYB family, also confer drought tolerance, implying that physiological pathways mediated by NFs also play an important role in regulating drought tolerance [25, 26].

NAC domain-containing transcription factors constitute both ABA-dependent and ABA-independent pathways. The *RD26* gene is induced by ABA and drought stress,

implying that RD26 may function in an ABA-dependent manner. A microarray analysis has shown that the RD26 transcription factor largely affects expression of stress- and ABA-responsive genes. As a result, while the 35S:*RD26* transgenic plants are highly sensitive to ABA, loss-of-function mutants are insensitive to ABA [27].

In contrast, three NAC transcription factors, ANAC019, ANAC055, and ANAC072, have been identified as regulators of drought stress responses independent of ABA. The transcription factor genes are highly induced by drought stress and high salinity. They bind directly to the NAC recognition sequence motif (CATGTG) existing both in the 63-bp region of the *EARLY RESPONSIVE TO DEHYDRATION STRESS 1* (*ERD1*) gene promoter [28] and in the diverse stress-responsive gene promoters. Overexpression of *ANAC019*, *ANAC055*, or *ANAC072* leads to upregulation of stress-responsive genes and enhanced drought tolerance [28]. The ZINC FINGER HOMEODOMAIN 1 (ZFHD1) transcription factor also binds directly to the *rps1* site 1-like sequence (CACTAAATTGTCAC) in the *ERD1* gene promoter. The *ZFHD1* gene is induced by ABA, drought, and high salinity. Overexpressing the *ZFHD1* gene induces expression of several stress-responsive genes and enhanced resistance to drought stress [29]. Notably, the ANAC and ZFHD1 transcription factors interact with each other to activate the *ERD1* gene, constituting an ABA-independent signaling pathway.

Consistent with this, several genes are still induced by drought stress in the *aba* biosynthetic mutants, suggesting that ABA-independent pathways also play a role in drought stress responses [14]. The *CBF1*-overexpressing transgenic plants are also resistant to drought and salt stresses, suggesting that genes containing the DRE in their promoters may also be regulated by drought and salt stresses [7]. The *COR* genes induced by CBFs may also play a protective function under drought stress conditions. While the *DREB1* genes are induced specifically by cold stress, the *DREB2* genes are regulated by drought and high salt stresses but not by cold stress [14]. The *DREB2* genes may confer drought tolerance by inducing the genes containing the DRE motifs in their promoters [14].

20.3.2
Salt Stress

High salinity causes hyperosmotic and hyperionic stresses to plants. In terms of water potential, basic physiology of high salinity and drought stress is highly overlapped with each other. Therefore, research on salt stress responses has been oriented to those ion-specific stress responses that repair cellular imbalance of K^+ and Na^+ ions. Briefly, various ion pumps, transporters, and channels are involved in maintaining sodium ion homeostasis. A representative example is the salt overly sensitive (SOS) pathway. It participates in the regulation of ion homeostasis. The *SOS3* gene encodes a CALCINEURIN B-LIKE (CBL) protein, which senses cytosolic Ca^{2+} by directly binding to ions. The SOS2 protein is serine/threonine protein kinase, which is activated by SOS3 in a calcium-dependent manner. The SOS1 protein is a Na^+/H^+ antiporter and is phosphorylated by the SOS3–SOS2 complex, which eventually causes reduction of cytosolic Na^+ concentration [9, 30].

Transcriptional regulation of salt stress responses is quite similar to that of drought stress responses [7, 8]. Accumulation of ABA is induced under high salinity and subsequently activates ABA-responsive genes. Interaction of bZIP transcription factors with the ABRE *cis*-acting elements is a major regulatory scheme in the salt stress responses. Several MYB and MYC transcription factors, which play roles in drought stress responses, are also involved in salt stress responses [31].

Some transcription factors have been identified as key players in salt stress-specific responses. It has been reported that A NAC domain-containing transcription factor regulates salt stress response and lateral root development through signaling interaction with growth hormones. A gene encoding the NAC2 transcription factor is highly induced by high salinity. It has been proven that it is also influenced by ethylene and auxin signalings [32]. Notably, ETHYLENE INSENSITIVE 2 (EIN2) converges ethylene and auxin signals and positively regulates the *NAC2* gene [32]. As a result, *NAC2* expression is completely blocked in the *ein2* mutant. The NAC2-mediated promotion of lateral root formation reflects an adaptive stress response under salt salinity [32].

A few WRKY transcription factors have been reported as specific mediators of salt stress responses. Expression of the *WRKY25* and *WRKY33* transcription factor genes is induced by high salt. Analysis of the induction patterns indicates that *WRKY33* expression partially depends on ABA but is independent of SOS. Transgenic plants overexpressing either the *WRKY25* or the *WRKY33* gene are tolerant to high salt stress. In contrast, the *wrky25wrky33* double mutant is sensitive to high salinity [33].

The AP2/ERF transcription factors are also involved in salt stress responses. Transcription of the *CBF4/DREB1D* gene is induced by osmotic stress [14]. Because the CBF/DREB transcription factors bind to the CRT/DRE sequence, it seems that most of the CBF/DREB-mediated signals are converged at the gene promoters containing the CRT/DRE sequence [34]. The *DWARF AND DELAYED FLOWERING 1* (*DDF1*) gene encoding an AP2 transcription factor, belonging to the CBF/DREB1 subfamily, regulates primarily the *GA 2-OXIDASE 7* gene (*GA2ox7*) [35]. The GA2ox7 protein is a C_{20}-GA deactivating enzyme. The *DDF1* gene is induced strongly by high salt stress, which subsequently upregulates the *GA2ox7* gene by directly binding to the DRE-like motifs (GCCGAC/ATCGAC) possibly via an ABA-independent manner [35]. This signaling supports the growth repression under high salinity. In addition to *GA2ox7*, other salt stress-responsive genes, such as *RD29A*, *COR15A*, and *KIN*, are also upregulated in 35S:*DDF1* transgenic plants, conferring resistance to salt stress [35].

20.3.3
Cold Stress

Temperate plants develop freezing tolerance through sustained experience of low but nonfreezing temperatures [9]. Cold acclimation responses include rapid induction of many transcriptional activator genes, such as those encoding the CBFs/DREBs transcription factors, reduction of growth, and modulation of metabolic activity [36, 37]. Changes in transcriptome have been intensively investigated and analyzed. The *CBF1/DREB1B*, *CBF2/DREB1C*, and *CBF3/DREB1A* genes, which play a

central role in cold acclimation process, have been identified through this approach [38, 39]. These genes are rapidly induced upon exposure to cold within 15 min, and constitutive expression of these genes enhances freezing resistance [38, 40]. The DREB/CBF transcription factors directly regulate the *COR* genes via the DRE *cis*-acting elements in respond to cold stress independent of ABA. *Arabidopsis COR* genes (*COR6.6*, *COR15a*, *COR47*, and *COR78/RD29a*) encoding LEA-like proteins are critical for development of freezing tolerance and cold acclimation.

INDUCER OF CBF EXPRESSION 1 (ICE1) is a MYC-like bHLH transcription factor and binds directly to the MYC recognition sequence present in the *CBF3* gene promoter [41]. An R2R3-type MYB transcription factor MYB15 is also involved in the regulation of the *CBF3* gene through interaction with ICE1 [42]. It is highly possible that other bHLH and MYB transcription factors regulate the *CBF1* and *CBF2* expression. Transcriptional regulation of *CBF*s by ICE1 is not ordinary. Considering the rapid induction of *CBF*s upon exposure to cold, ICE1 should be present in the nucleus under normal growth condition and its conformation or interaction with other binding partners would be modified in response to cold, which in turn regulates expression of the *CBF3* and *COR* genes in inducing freezing tolerance [36]. To date, the ICE1–CBF–COR regulon is a major cold stress signaling pathway, which regulates a wide array of cold-responsive genes and thus induces freezing tolerance (Figure 20.2). Sensors, which are responsible for activation of the major pathway remain to be elucidated.

The *CBF* genes are also regulated by negative regulators. The ZAT12 transcription factor is a zinc finger protein that contains EAR-motif sequence that may function as a transcriptional repression domain [43, 44]. The ZAT12 transcription factor is

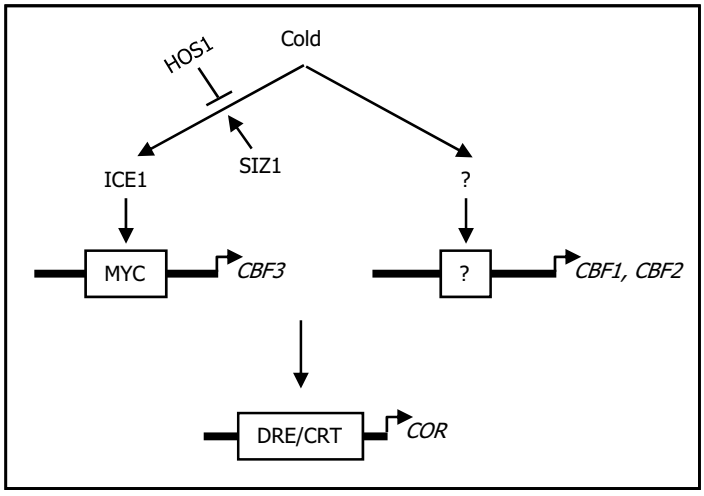

Figure 20.2 Transcriptional regulatory networks in cold stress signaling. HOS1 E3 ligase-mediated process regulates a key transcription factor ICE1 at the protein level. Protein sumoylation is also linked to the ubiquitination pathway.

involved in regulation of the *CBF* expression in response to cold stress. In addition, constitutive expression of *CBF2* suppresses the expression of *CBF1* and *CBF3*, establishing a negative feedback loop [45].

Because of its importance, CBF orthologues have been identified and functionally characterized in other plant species. *Arabidopsis* transgenic plants overexpressing the rice *OsDREB1A* gene exhibit phenotypes similar to those overexpressing the *Arabidopsis DREB1A* gene, indicating that this transcription factor is conserved in both monocot and dicot [46]. *Lycopersicum esculentum* contains three *CBF* genes in a tandem array [47]. Constitutive expression of *LeCBF1* in *Arabidopsis* also confers freezing tolerance and regulates cold-responsive genes [47]. *Brassica napus* also has four orthologues of the *Arabidopsis CBF* genes. Wheat and rye are suspected to have the AP2 transcription factor, which is homologous to *Arabidopsis* CBF, suggesting that the CBF regulon is conserved in diverse plant species [48, 49].

Several cold-responsive genes do not contain the DRE in their promoters, suggesting that *cis*-acting elements other than the DRE are also involved in cold-responsive gene expression [50]. Moreover, constitutive expression of *CBF* genes does not lead to full cold acclimation in *Arabidopsis*, supporting the view that additional signaling pathways participate in cold acclimation and responses. The *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 9* (*HOS9*) gene encodes a homeodomain-containing transcription factor localized in the nucleus. The *hos9-1* mutant shows reduced freezing tolerance without alterations in *CBF* gene regulation. The *HOS9* gene is unaltered by cold, suggesting that it may have a role in basal freezing tolerance response [51]. Although molecular function is unknown, the ESKIMO1 (ESK1) protein also regulates freezing tolerance by inducing proline accumulation. Loss-of-function mutant of *ESK1* exhibits enhanced and constitutive freezing tolerance. Genes affected by the *esk1* mutation are largely independent of CBF pathway, supporting the view that CBF-independent pathways are also involved in cold adaptation and freezing tolerance. Transcription factors in ABA-dependent pathways have also been investigated. It seems that bZIP, MYC, and MYB transcription factor family members may participate in the regulation of cold-responsive gene expression [13, 52–54].

Overall, cold-regulated genes include those encoding COLD-REGULATED (COR), LOW-TEMPERATURE-INDUCED (LTI), COLD-INDUCIBLE (KIN), and EARLY DEHYDRATION RESPONSIBLE (ERD). These genes are regulated through interactions of the transcription factors with *cis*-acting elements in the gene promoters. Interactions between DREB/CBF transcription factors and DRE/CRT elements establish an ABA-independent pathway. In contrast, bZIP interactions with ABRE, MYB interactions with its recognition sequence (TGGTTAG), and MYC interactions with its recognition sequence (CACATG) constitute ABA-dependent pathways.

20.3.4 Heat Stress

Ambient temperatures above optimal ranges are sensed as heat stress in eukaryotes. Heat stress disturbs cellular homeostasis and causes severe growth retardation, arrested development, and death in many cases. Most of the higher plants are unable

to survive from extended exposure to temperatures above 45 °C [55]. As plants are sessile, they have evolved a complex set of defensive mechanisms against stressful high temperatures, which minimizes damage and protects cellular homeostasis.

Prior treatment with high temperatures often induces thermotolerance to lethal temperatures. This heat acclimation involves accumulation of a group of heat shock proteins (HSPs), which act as molecular chaperones by facilitating refolding of denatured proteins and removing misfolded proteins [56, 57]. Induction of HSP accumulation is regulated primarily at the transcriptional level by heat shock transcription factors (HSFs), which bind specifically to the heat shock element (HSE) "GAANNTTC" existing in the promoters of *HSP* genes [58].

HSFs are central components in heat stress signaling that sense temperature changes to regulate genes involved in heat shock response. Similar to many other transcription factors, HSFs have a modular structure. The highly conserved N-terminal DNA binding domain is characterized by an HLH motif and an adjacent oligomerization domain (OD) having a hydrophobic heptad repeat pattern [59, 60]. These structural components are also important for heat stress-dependent activation that converts inactive HSF monomers to trimeric forms, which bind specifically to the HSE element in the promoters of HSF-responsive genes [61].

Plants contain a small family of *HSF* genes. More than 20 HSF members have been defined in *Arabidopsis* and tomato [59, 60]. This is in contrast to what has been observed in other eukaryotes. For example, vertebrates have only three HSF members, and yeast and *Drosophila* have a single HSF in each case [62]. On the basis of the structural details of the oligomerization domains, plant HSFs are grouped into three conserved evolutionary classes: A, B, and C. In tomato, HSFA1a and HSFB1 belong to class A, which form a regulatory network acting as master regulators of heat stress-responsive genes [60, 62]. The *HSFA1a* gene is constitutively expressed and mediates heat stress-induced expression of *HSFA2* and *HSFB1* [63]. HSFA1a also functions as a nuclear retention factor and as a coactivator of *HSFA2* by forming HSFA1a–HSFA2 heterooligomeric complexes.

It is notable that HSFs belonging to class B or class C have no evident activities as transcription activators of their own. HSFB1 has been identified as a coactivator cooperating with class A HSF members and other transcription factors [64–66].

Genome-wide transcriptome analysis of *HSFA1a*, *HSFA1b*, and *HSFA2* knockout mutants in *Arabidopsis* suggests that HSFA1a and HSFA1b play important roles in the initial phase of heat stress response, but HSFA2 functions under prolonged heat stress conditions and in the later recovery phase [67–69]. The heat stress-induced expression of *HSFA2* in *Arabidopsis* is uninfluenced by HSFA1a or HSFA1b [67]. The *HSFA2* gene is also induced by high light intensity and H_2O_2 [69]. It is also closely related to the regulation of *ASCORBATE PEROXIDASE 2* (*APX2*) encoding a key enzyme in oxidative stress response, indicating that HSFA2 plays diverse roles under various environmental stresses. The heat stress induction of *Arabidopsis HSFA3* is regulated directly by DREB2A, a transcription factor functioning in drought stress responses [70]. Accordingly, the DRE has been identified in the promoters of a cluster of heat-inducible genes [71]. It has been

proven that the heat-induced DREB2A transcription factor binds to the DRE present in the *HSFA3* gene promoter [72, 73].

Additional heat-inducible transcription factors are also involved in thermotolerance. Under heat stress conditions, the *NF-X1* (*NUCLEAR TRANSCRIPTION FACTOR X-BOX BINDING 1*) gene shows an induction pattern similar to that of genes having DREs in their promoters. The NF-X1 transcription factor promotes tolerance to heat and salt stresses [71]. The transcriptional coactivator MBF1c (MULTIPROTEIN BRIDGING FACTOR 1C), which is involved in multiple stress responses, accumulates rapidly after exposure to heat stress. In contrast, MBF1c is not required for the expression of the *HSFA2* gene and other *HSP* genes [74]. The *Arabidopsis bZIP28* gene encoding a putative membrane-tethered transcription factor is induced in response to heat stress, and a *bZIP28* null mutant exhibits a heat-sensitive phenotype [75]. Upon exposure to heat, the bZIP28 protein, which is localized to the endoplasmic reticulum membranes, undergoes proteolysis to release a nuclear form. Although direct target genes have not been determined, heat-inducible expression of a gene *Bip2* encoding an endoplasmic reticulum chaperone and a small HSP gene *HSP26.5-P* is altered in the mutant.

20.4
Regulation of Transcription Factor Activities

Transcription factor should act in appropriate tissues or cells at appropriate times by directly binding to target DNA sequences. They also interact with other regulatory factors, such as transcriptional regulators. Temporal and spatial regulation of transcription factor activities is also important for understanding molecular mechanisms underlying abiotic stress signaling and responses. Transcription factors are regulated at multiple steps, such as gene transcription, posttranscriptional RNA processing, posttranslational modification, protein–protein interactions, and controlled protein turnover.

20.4.1
Transcriptional Control

Gene transcription is a primary step to regulate transcription factor activities. It usually determines tissue-specific expression, stage-dependent expression, and signal-inducible expression. Gene regulation under diverse environmental conditions is mediated by intensive transcriptional regulatory cascades [76]. Early-response genes are induced rapidly within minutes after exposure to stress conditions. Notably, a large portion of the early-response genes encodes transcription factors, suggesting that transcriptional regulation is a central regulatory scheme in earlier steps of stress adaptive responses [8, 10]. Transcriptional regulation also facilitates additional steps of gene regulations, such as posttranscriptional, translational, and even posttranslational control of transcription factor genes.

20.4.2 Posttranscriptional Modification

Posttranscriptional regulation is exerted at the RNA level. Recent studies suggest that it plays an essential role in acquisition of abiotic tolerance. Posttranscriptional regulation is exerted at variable steps, such as alternative splicing, controlled mRNA processing, mRNA silencing, nucleocytoplasmic transport, and accessibility to translational apparatus. In this section, we more focus on alternative splicing and mRNA silencing.

RNA splicing is the excision of intron sequences from pre-mRNA mediated by spliceosome. Alternative splicing (AS) generates multiple mRNAs from a single primary transcript through alternative selection of splice sites in the pre-mRNA [77, 78]. It has been reported that 95% of human genes having multiple exons undergo AS [79]. In plants, over 35% of *Arabidopsis* and rice genes are considered to be alternatively spliced [80–82], indicating that AS is widespread in eukaryotes. AS occurs through exon skipping, alternative selection of 5′ and 3′ splice sites, and intron retention. Such molecular events produce a small group of protein isoforms, which possess differential activities. Alternatively, some of them are degraded through nonsense-mediated decay (NMD) [83, 84]. Exon skipping is the most frequently observed AS mechanisms. In contrast, intron retention is rarely observed in animals. It is notable that intron retention is the most frequently occurring AS event in *Arabidopsis* and rice [85, 86].

It seems that AS affects preferentially a certain class of genes that is mostly involved in signal transduction or encodes specific enzymes, receptors, and transcription factors [85, 87]. The wheat *WDREB2* gene, an *Arabidopsis DREB2* homologue, produces three differential transcripts through exon skipping under stress conditions. The three isoforms have different accumulation patterns, and relative ratio of the transcript isoforms is regulated via an ABA-dependent pathway under drought and salt stresses and an ABA-independent pathway at low temperatures [88]. A subgroup of MYB transcription factor genes in *Arabidopsis* and rice produces alternatively spliced transcripts, accumulation of which is influenced by various phytohormones and stress signals. AS of the *MYB* genes results in three (rice) or four (*Arabidopsis*) distinctively spliced transcripts for each gene, producing putative proteins differing by numbers of MYB repeats and probably by their binding affinities to gene promoters [89]. Durum wheat genes encoding a putative ribokinase and a C3H2C3 RING finger protein undergoes AS, in which a subset of introns are retained under stress conditions [90].

AS of genes encoding nuclear splicing factors is also influenced by abiotic stresses. Nineteen genes encoding serine-/arginine-rich proteins, which are classified as RNA binding proteins with a role as a splicing regulator in eukaryotes, have been identified in *Arabidopsis*, and most of them undergo AS in response to environmental stimuli [91, 92]. *STABILIZED1* (*STA1*), a gene encoding a pre-mRNA splicing factor, is induced under cold stress conditions in *Arabidopsis*. The *sta1-1* mutant has defect in the splicing of *COR15A* mRNA, resulting in hypersensitivity to chilling and salt stresses and ABA [93]. Regulation of AS of a specific gene and subsequent regulation

of AS of other genes may contribute to enhancement and amplification of abiotic stress signal transduction cascades.

Small noncoding RNAs consisting of 20–25 nucleotides (nts), such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), emerge as essential players in posttranscriptional control of gene expression. They are processed from double-stranded RNA (dsRNA) precursors by specific RNases belonging to the DICER-LIKE (DCL) family. One strand of the processed duplex form is then combined with the AGONAUTE (AGO) protein that form RNA-induced silencing complex (RISC). The RISC ribonucleoprotein complex recognizes target mRNAs that contain a complementary sequence to the small RNA and subsequently silences them by either guiding RNA degradation or repressing translation of target mRNAs [94–96].

A large group of small RNAs has been identified primarily by computational identification of small RNAs and their corresponding mRNA targets [97, 98]. Recently, microarray-based large-scale analysis and computational transcriptome analysis have shown that numerous miRNAs are differentially accumulated in response to cold, dehydration, high salinity, and nutrient starvation [99, 100]. Notably, the genetic loci encoding these miRNAs contain stress-related *cis*-acting elements in their promoter regions, suggesting that RNA silencing by small RNAs is an important gene regulatory scheme in plant stress responses.

Although most small RNAs target transcription factor genes, those functioning in plant responses to abiotic stress and nutrient deficiency frequently regulate genes encoding enzymes and transporters involved in plant metabolism [101, 102]. In *Arabidopsis*, miR393 negatively regulates several genes encoding F-box protein, such as *TIR1* and *AFB*s, to acquire resistance to pathogen infection [103]. miR393 abundance is increased by diverse abiotic stress conditions, and accordingly transcript levels of its targets are reduced under identical conditions [98]. While abiotic stress-mediated miRNA regulation is widely documented, miRNA regulation of transcription factor genes is still elusive.

An additional class of small RNAs, referred to as natural antisense transcripts (NAT)-generated siRNAs (nat-siRNAs), has recently been proven to function in plant stress responses [104]. The nat-siRNAs are produced from dsRNA formed by intermolecular association of NATs and complementary transcripts. For example, accumulation of proline under salt stress conditions is regulated by a nat-siRNA-mediated pathway. The *P5CDH* (*D-PYRROLINE-5-CARBOXYLATE DSHYDROGENASE*) gene encoding proline catabolic enzyme is constitutively expressed under normal conditions [104]. The *SRO5* (*SIMILAR TO RADICAL-INDUCED CELL DEATH ONE 5*) gene is transcribed from the identical *P5CDH* locus, but in an opposite direction. Salt stress induces expression of both genes, leading to accumulation of partially complementary dsRNA formed by base pairing of the two transcripts. This dsRNA is subsequently cleaved by DCL, generating two siRNAs consisting of 24 nts and 21 nts. These siRNAs trigger silencing of the *P5CDH* gene by mRNA cleavage and lead to partial inhibition of proline catabolism. This salt-dependent control of proline accumulation via the nat-siRNA and P5CDH pathways allows better tolerance to salt stress. More than 2000 NATs are predicted by bioinformatic analyses in *Arabidopsis* [105, 106]. Therefore, it is expected that

nat-siRNA-mediated regulation is a genome-scale mechanism modulating environmental stress responses in plants.

20.4.3 Posttranslational Modification

Several transcription factors require posttranslational modifications for their activation. Simple overexpression of these transcription factor genes does not induce any physiological responses. For example, transgenic plants overexpressing membrane-bound transcription factor (MTFs) genes do not exhibit phenotypic alterations [17, 107]. It has been shown that these transcription factors should be modified posttranslationally to obtain specific activities.

20.4.3.1 Membrane-Bound Transcription Factors

Regulation of subcellular localization is a fundamental mechanism that activates transcription factors. Transcription factors exert its activity in the nucleus. However, some transcription factors are present in dormant forms in the cytoplasm. Biochemical modifications and interactions with certain partners induce their nuclear translocation [108, 109].

The plasma membrane is the primary site that perceives external signals, such as signal peptides, growth hormones, and ligands. Many membrane-bound proteins are intimately related to signal perception and signaling transduction from the plasma membranes to the nucleus. It has been reported that a group of transcription factors are membrane-associated in plants. The MTFs are stored in dormant forms in association with intracellular membranes, such as the plasma membranes, nuclear membranes, and ER membranes [110]. Upon stimulation by intrinsic and extrinsic signals, they are proteolytically activated via either regulated intramembrane proteolysis (RIP) mediated by membrane-bound protease or regulated ubiquitin/proteasome-dependent processing (RUP) modulated by proteasomic activity. The processed MTF forms are translocated into the nucleus, where they regulate expression of target genes as ordinary transcription factors (Figure 20.3).

Several *Arabidopsis* NAC and bZIP transcription factors have been shown to be associated with the plasma membranes and ER membranes, respectively [110]. These MTFs are type II membrane proteins with their N-termini oriented toward the cytoplasm. They are activated by specific membrane-associated proteases, such as the SITE-1-PROTEASE (S1P) and S2P, via the RIP mechanism [110]. Notably, most of the MTFs are involved in diverse abiotic stress responses. A genome-wide screening has revealed that at least 13 members of the NAC transcription factors are membrane-tethered, which are collectively termed as NTLs (*NT*M1-*L*ike), in *Arabidopsis* [111]. These transcription factors do not induce any phenotypic alteration when full-size forms are overexpressed. Instead, overexpression of processed, nuclear forms induces specific physiological changes, indicating that release from the membrane is critical for their activities. Roles of several NTLs have been explored in plant responses to high salinity, osmotic stress, and cold. NTL6 is proteolytically activated by cold-induced membrane rigidification, and the activated NTL6 form regulates

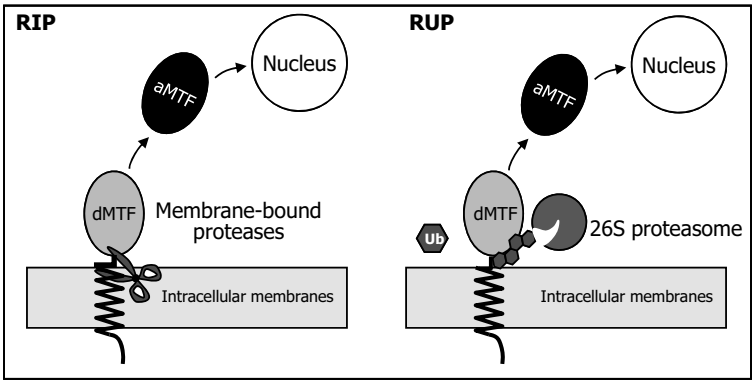

Figure 20.3 Proteolytic activation schemes of membrane-bound transcription factors. The membrane-bound, dormant MTF (dMTF) is released from the membranes by either RIP or RUP. The activated MTF (aMTF) enters the nucleus. Adapted from Seo *et al.* [110].

directly a subset of *PATHOGENESIS-RELATED* (*PR*) genes [112]. NTL8 mediates salt regulation of flowering initiation and seed germination [113, 114]. In addition, NTL9 regulates leaf senescence in response to osmotic stress [115]. Several bZIP MTFs, including bZIP60 and bZIP28, have been shown to play a role in ER stress responses [116–118]. bZIP17 participates in plant responses to salt stress. It is now widely accepted that sequestration of transcription factors from the nucleus provides a way of quick transcriptional responses to environmental fluctuations by skipping the transcription and translation steps.

Extensive genome-wide analyses of the *Arabidopsis* and rice genomes have predicted that at least 190 TFs, constituting approximately 10% of the all plant transcription factors, are physically associated with intracellular membranes [111]. These observations support the hypothesis that membrane biology is a critical component of transcriptional regulatory networks in plants.

20.4.3.2 **Phosphorylation**

Biochemical modifications greatly alter transcription factor activities. Phosphorylation is recognized as a major posttranslational modification of transcription factors that regulate their subcellular localization and transcriptional activation [119–121].

Snf1-related protein kinase (SnRK) is a representative serine/threonine protein kinase family, which positively regulates ABA-dependent signaling [122]. The *Arabidopsis* genome contains 38 SnRKs, of which SnRK2 consists of 10 members [122]. SnRK2.2 and SnRK2.3 redundantly regulate ABA signaling, such as seed germination and seedling growth, by phosphorylating ABFs/AREBs [123]. In addition, SnRK2.6 (OST1/SnRK2E) is involved in regulation of stomatal aperture and transpiration, and SnRK2.8 (SRK2C/OSKL4) regulates drought tolerance without affecting stomatal aperture [73, 124]. As a result, transgenic plants overexpressing either SnRK2.6 or SnRK2.8 exhibit enhanced drought tolerance [73, 124]. Consistent with this, transgenic plants expressing a constitutively phosphor-

ylated AREB1 show ABA-responsive phenotypes even without exogenous application of ABA, supporting that protein phosphorylation is a crucial regulatory scheme of osmotic stress resistance [17].

SnRK2-mediated osmotic stress responses are further modulated by ABA perception machinery. Type 2C protein phosphatase (PP2C) induces stomatal closure and drought stress responses by inactivating SnRK2s [125]. Expression of more than 90% of ABA-responsive genes is abolished in the *abi1-1* mutant, supporting a central role of PP2C in ABA signaling [126]. Interactions of PP2Cs with SnRK2s have been studied using potential ABA receptors [127, 128]. The ABA-bound PYR1 (PYROBACTIN RESISTANCE PROTEIN 1) protein disrupts the interaction between SnRK and PP2C by directly docking the active site of PP2C. Inhibition of the PP2C-mediated dephosphorylation of SnRK by PYR1 activates downstream events of ABA signal transduction cascades [127–130].

Several calcium-sensing proteins also participate in ABA signaling. The CBL-interacting protein kinase CIPK15/PKS3 interacts with ABI1 and ABI2, which play a central role in ABA signaling [131]. In addition, the kinase also phosphorylates the AP2/ERF-type transcription factor ERF7 that negatively modulates ABA signaling [132]. The calcium-dependent protein kinases (CDPKs), which constitute 34 members, have also been identified as ABA signaling regulators. The CPK32 kinase interacts with the ABF4 transcription factor, and overexpression of *CPK32* leads to ABA-hypersensitive phenotypes [133]. Likewise, the CPK4 and CPK11 activities are also stimulated by ABA. The *cpk4 cpk11* double mutant shows reduced ABA and stress responsiveness and reduced resistance to salt stress. In contrast, transgenic plants overexpressing either *CPK4* or *CPK11* exhibit slightly enhanced resistance to salt and drought stresses. The CPK4 and CPK11 kinases phosphorylate the ABF1 and ABF4 transcription factors and positively regulate Ca^{2+}-mediated ABA signaling [134].

20.4.3.3 **Ubiquitination**

Ubiquitin is widely known as a ubiquitous protein modifier that guides target proteins to proteasome-dependent degradation. Proteins designated for degradation are modified by covalent attachment of ubiquitin polymers [135]. Ubiquitinated protein is degraded by the 26S proteasome [136]. For protein ubiquitination, a series of enzymes function in sequence. The ubiquitin-activating enzyme (E1) catalyzes formation of thioester bond between ubiquitin and itself using ATP as energy source. The activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2). Finally, the ubiquitin ligase (E3) facilitates formation of isopeptide bond between the activated ubiquitin and the protein substrates [136]. The E3 ligase interacts with both E2 enzyme complex and substrates to determine target specificity [137]. The majority of the enzymes involved in the ubiquitination process consist of E3 ligases, and a large portion of E3 ligases is the REALLY INTERESTING NEW GENE (RING) type [138]. Ubiquitination-mediated regulation of transcription factors is frequently found in plant response to abiotic stresses.

The SALT- AND DROUGHT-INDUCIBLE RING FINGER 1 (SDIR1) E3 ligase that is involved in drought and salt stress signaling and depends on ABA [139]. The *SDIR1* gene is induced by drought and salt stresses, and its overexpression leads to ABA- and

osmotic stress-hypersensitivity, which results in enhanced drought tolerance. In addition, expression of ABA- and stress-responsive genes is profoundly altered in loss-of-function mutants of *SDIR1* that exhibit ABA insensitivity. Although its direct targets have not been identified, SDIR1-mediated signaling affects expression of the *ABI3* and *ABI5* genes and resistance to drought and salt stresses [139].

Among the four ABI3-INTERACTING PROTEINS (AIPs) identified through yeast two-hybrid assays [140], AIP2 functions as a ubiquitin E3 ligase that degrades ABI3 [141]. As inferred from the interactions between ABI3 and AIP2, protein stability of the ABI3 transcription factor is increased in the *aip2-1* mutant but decreased in *AIP2* overexpressors. Accordingly, the *aip2-1* mutant, in which the ABI3 transcription factor accumulates, exhibits hypersensitive responses to ABA, supporting that AIP2 negatively regulates ABI3-mediated ABA signaling [141].

The ABI5 transcription factor is also regulated by ubiquitin-mediated degradation in nuclear bodies [138]. The RING-type E3 ligase KEEP ON GOING (KEG) negatively regulates the ABI5 transcription factor. While the ABI5 proteins accumulate in loss-of-function mutant of *KEG*, transgenic plants overexpressing *KEG* are insensitive to ABA and high salt. In the absence of ABA, KEG maintains the ABI5 transcription factor at a low level. However, in the presence of ABA, the KEG protein is degraded through autoubiquitination, which increases ABI5 protein stability and activity [142]. Notably, protein phosphorylation accelerates degradation of the KEG protein in response to ABA [142].

The DREB2A transcription factor is negatively regulated by DREB2A-INTERACTING PROTEIN 1 (DRIP1) and DRIP2. Overexpression of *DRIP1* leads to delayed induction of DREB2A-regulated genes. In contrast, expression of genes regulated by DREB2A and drought stress is greatly induced in the *drip1-1drip2-1* double mutant. DRIP1 is a potential C3HC4 RING-type E3 ligase and mediates ubiquitination of the DREB2A protein, further supporting the significance of ubiquitination in ABA responses [143].

The ubiquitin/26S proteasome pathway is also implicated in cold stress signaling. The *HOS1* gene encodes a RING finger protein acting as a ubiquitin E3 ligase enzyme. HOS1 is present in the cytoplasm under normal growth conditions. However, it is translocated to the nucleus in response to cold. Notably, HOS1 physically interacts with ICE1 in the nucleus [144]. The HOS1 protein degrades the ICE1 transcription factor through the ubiquitination process and fine-tunes expression of the *CBF/DREB1* gene under cold stress. As a result, cold stress-responsive genes are induced in the *hos1* mutant, and cold stress resistance is enhanced. On the other hand, overexpression of *HOS1* suppresses the *CBF* gene and reduces freezing tolerance, indicating that HOS1-mediated ubiquitination is a crucial negative regulation mechanism in cold stress signaling [144].

20.4.3.4 **Sumoylation**

Sumoylation is emerging as one of the major posttranslational modification processes. Small ubiquitin-related modifier (SUMO) is reversibly conjugated to protein substrates, and SUMO conjugation/deconjugation is conserved in eukaryotes [145]. Sumoylation is very similar to ubiquitination process. Indeed, E1, E2, and E3

enzymes, analogous to those mediating ubiquitination, are involved in sumoylation [146]. Sumoylation regulates subcellular localization and enzymatic activity of substrate proteins and functions in innate immunity and DNA repair. Notably, SUMO conjugation also modulates activities of transcription factors in response to developmental and environmental cues [145].

Among the 8 SUMO isoforms, only SUMO1 and SUMO2 isoforms are conjugated to target proteins in response to environmental stresses, and stress-mediated SUMO conjugation is brought mainly by AtSIZ1 [147–150]. AtSIZ1-mediated ICE1 sumoylation induces expression of the *CBF3/DREB1A* gene and thus enhances freezing tolerance in *Arabidopsis*. Consistent with this, the *siz1-2* and *siz1-3* mutants exhibit freezing-sensitive phenotypes. Sumoylation of ICE1 at K393 inhibits ubiquitination that is possibly mediated by the HOS1 E3 ligase [150]. Therefore, AtSIZ1-mediated sumoylation stabilizes the ICE1 protein and thus confers freezing tolerance [145].

AtSIZ1 also negatively regulates ABA signaling. Expression of ABA-responsive genes containing the ABREs in their promoters is upregulated in the *siz1-2* and *siz1-3* mutants that exhibit hypersensitive responses to ABA [151]. ABA hypersensitivity of the *siz1* mutants is caused by accumulation of a bZIP transcription factor ABI5. However, the hypersensitive response is compromised in the *siz1 abi5-4* double mutant, indicating that SIZ1 negatively regulates ABI5-mediated ABA signaling. The SIZ1-mediated ABI5 sumoylation at K391 inactivates the transcription factor activity independent of AFP and KEG-mediated degradation [151].

Sumoylation and ubiquitination regulate target proteins either in a cooperative manner or in a competitive manner to maintain protein function and activity to an appropriate level. Unlike ubiquitin-mediated degradation, reversible mechanism of sumoylation/desumoylation serves as a switch system in regulating transcription factor activities under stress conditions [146]. Accordingly, SUMO proteases, which cleave the linkages between SUMO and substrates, have been identified as stress response regulators [152]. The OVERLY TOLERANT TO SALT 1 (OTS1) and OTS2 proteins are such SUMO proteases, which participate in salt stress responses. While the *ots1 ots2* double mutant is sensitive to high salinity because of high-level accumulation of SUMO-conjugated proteins, *OTS1* overexpression leads to an enhanced salt tolerance [152]. Although identification of OTS1/2 substrates is elusive, OTS1/2-mediated pathway is an attractive target for genetic engineering of salt-tolerant crops.

20.4.4
Protein–Protein Interactions

Dynamic protein dimerization plays a critical role in the regulation of transcription factor activities. A number of transcription factors function as dimers to assure DNA binding specificity. For example, bZIP transcription factors form both homodimers and heterodimers via the coiled coil motifs [153, 154]. DNA binding affinity and transcriptional regulation activity vary within a transcription factor family. Thus, dynamic formation of homodimers and heterodimers modulates specific activities and functional diversities of transcription factors [154].

This regulatory mechanism is also observed widely in transcription factors mediating abiotic stress responses. The AtMYC2 and AtMYB2 transcription factors coordinately regulate the *RD22* gene, supporting that dimer formation of transcription factors is important for controlling the target genes [13].

NAC transcription factors, such as ANAC019, ANAC055, and ANAC072, regulate stress-responsive genes by binding directly to NAC recognition sequences in the gene promoters. As a result, overexpression of individual ANAC transcription factors affects expression of stress-responsive genes and induces drought tolerance. Although the *ERD1* gene promoter contains NAC recognition sequences, overexpression of the ANAC transcription factors does not fully induce the *ERD1* gene. It has been found that the ANAC transcription factors require the binding partner ZFHD1 for regulation of the *ERD1* gene [29]. Coordinate action of the ANAC and ZFHD1 proteins modulates expression of the *ERD1* gene to obtain developmental balances [29].

An exquisite regulatory scheme has recently been proposed to modulate transcription factor activities. A group of small peptides have limited sequence similarities to specific transcription factors. Although they possess protein dimerization motifs, they lack DNA binding domain or transcriptional regulation domain. It has been demonstrated that they form nonfunctional heterodimers with specific transcription factors and thus exclude the target transcription factor from DNA binding [155]. Genome-scale identification of small proteins having similar structural organization in the databases suggests that small peptide-mediated transcription control would be a regulatory mechanism widespread in plants [156]. To date, although such small proteins have not been reported in abiotic stress responses, it would certainly be a way of modulating transcriptional regulatory activities of a number of transcription factors functioning under stress conditions (Figure 20.4).

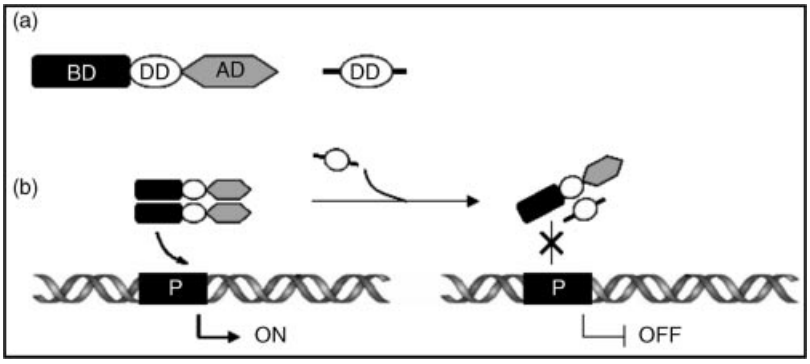

Figure 20.4 Small interfering peptide (siPEP)-mediated suppression of transcription factor activity. (a) Structural organization. The siPEP has a sequence similarity to a specific transcription factor. Although the former has the dimerization domain, it lacks other functional domains, such as DNA binding domain and activation domain, which are required for transcriptional regulation. (b) Competitive inhibition of transcription factor by siPEP. The siPEP forms nonfunctional heterodimers and thus competitively inhibits the formation of functional homodimers. The nonfunctional heterodimers possess reduced affinity to the promoter (P) sequence. Reprinted from [156] with kind permission by Chung-Mo Park.

20.4.5
Epigenetic Regulation

Genomic DNA is packed in a highly condensed structure in the nucleus. A group of nucleoproteins extensively interact with genomic DNA to establish the chromatin structure. Transcription factors bind target DNA sequences within chromatin to modulate gene expression. Chromatin structure, therefore, affects accessibility of transcription factor to their DNA target sites. Transcription factors easily access the naked DNA and open chromatin structure. In contrast, it is difficult for transcription factors to access the heterochromatin structure, suggesting the regulation of transcription factor activity by chromatin structure modification.

Changes in chromatin structure triggered by histone modifications constitute a crucial regulatory mechanism by which a wide spectrum of genes are expressed. Histone acetylation is dynamically regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone methylation is modulated by histone methyltransferases (HMTs) and histone demethylases (HDMs). Some of the histone modifying enzymes are involved in abiotic stress responses. The *Arabidopsis* GCN5 protein is an HAT enzyme that regulates cold stress responses [157]. GCN5 physically interacts with transcriptional machinery adapter proteins Ada2a and Ada2b. Remarkably, Ada2b-deficient mutants show enhanced freezing tolerance [158], suggesting that the HAT complex may directly or indirectly repress a signaling step that regulates freezing tolerance. It has also been reported that overexpression of the *Arabidopsis* HDAC homologue AtHD2C leads to enhanced resistance to salt and drought stresses [159].

The *hos15* mutant is hypersensitive to freezing stress. A WD40-repeat protein HOS15 is a component of protein complex involved in histone deacetylation and interacts specifically with histone H4, which results in deacetylation of histone H4. In the *hos15-1* mutant, acetylated histone H4 accumulates. The *RD29A* gene is greatly induced under cold condition possibly by hyperacetylation of histones. Additional genes influenced by the *hos15* mutation may also affect freezing sensitivity, supporting the view that histone acetylation/deacetylation is involved in cold adaptation responses [160].

In *Arabidopsis* H3, four acetylation sites (H3K9, H3K14, H3K18, and H3K23) and four methylation sites (H3K4, H3K9, H3K27, and H3K36) have been identified [161]. Such histone modification would also be widespread in plant stress responses. It has also been reported that distribution of H3 phosphorylation and H4 acetylation is dynamically regulated by abiotic stresses in various plant species, such as rye, barley, tobacco, and *Arabidopsis* [162, 163]. In addition, acetylation and methylation of histone H3 N-tail and nucleosome occupancy in the *RD29A*, *RD29B*, *RD20*, and *RAP2.4* genes are altered under drought stress conditions [164]. Under drought stress conditions, trimethylation of H3K4 and acetylation of H3K9 are induced, and certain genes are activated, and acetylation of H3K23 and H3K27 also occurs in the *RD29B* and *RD20* gene sequences [164, 165]. In addition, nucleosome density in the promoter regions of the *RD29A* and *RD29B* genes is lower than that

in the coding regions without nucleosome loss, facilitating rapid binding of transcription factors to the DRE and ABRE *cis*-acting elements [165]. Trimethylation of H3K27, which acts as a negative regulatory marker for gene expression, is decreased in cold-responsive genes, such as *COR15A* and *GALACTINOL SYTHASE 3* (*GOLS3*), under cold stress conditions, supporting the role of histone modification in abiotic stress responses [166].

The high-mobility group (HMG) proteins are the second most abundant proteins among the chromosomal proteins. They are involved in regulation of transcription and recombination. They also induce transient changes in chromatin structure [167, 168]. There are two groups of HMG proteins in plants [169]: HMGA having AT-hook DNA binding motif and HMGB having HMG-box domain. They interact with nucleosomes and transcription factors, supporting a role of HMG in gene transcriptional regulation [169]. Indeed, several plant HMGB proteins specifically interact with the bZIP and Dof transcription factors and assist their binding to target DNA sequences [170, 171].

There are 7 HMGB proteins in the *Arabidopsis* genome. At least a few of them are critical for stress responses [169]. The *HMGB1/2/3* genes are downregulated in response to high salinity, and transgenic plants overexpressing either *HMGB1* or *HMGB2* have alterations in seed germination under high salinity [169, 172]. However, molecular mechanisms underlying the HMGB regulation of stress responses have not been elucidated.

The switch (SWI)/sucrose nonfermenting (SNF) complex is a multisubunit DNA-dependent ATPase that is involved in chromatin remodeling [173]. It is also regulated by environmental stresses and subsequently regulates expression of stress-responsive genes. It has been observed that SWI3B interacts with PP2C and HYPERSENSITIVE TO ABA1 (HAB1) [174]. ABA inhibits binding of HAB1 to the gene promoters of *RD29B* and *RAB18*, and SWI/SNF chromatin-remodeling complex contributes to ABA responses. Consistently, the *swi3b* mutant exhibits insensitive responses to ABA [174].

20.5 Conclusions and Prospects

Understanding transcriptional cascades in stress signaling provides an excellent opportunity for stress-resistant crop production by genetic engineering. Transcription factors are most appropriate targets for genetic engineering to develop stress-resistant crops. A group of stress-responsive genes are coordinately regulated by introducing a transcription factor.

There are inevitable problems to be resolved in plant genetic engineering. In many cases, overexpression of stress-inducible genes leads to growth and developmental defects. To overcome this problem, inducible promoter would be an appropriate choice. Most of the genes are coordinately regulated by diverse signals through a web of signaling crosstalks, necessitating that multiple growth and developmental traits

are carefully considered before engineering genes of interests. One critical factor to be considered is gene promoter. Basically, any endogenous gene promoter in plants can be used to drive gene expression. Overexpression of the *DREB1A* gene in transgenic plants could activate a number of stress-tolerant genes, which results in enhanced resistance to drought, high salt, and freezing stresses. While the *DREB1A* gene expression driven by the cauliflower mosaic virus (CaMV) 35S promoter results in severe growth retardation under normal growing conditions, expression of a *DREB1A* gene expression construct fused to the *RD29A* gene promoter only slightly influences plant growth [175].

Heterologous expression of useful genes may confer advantages in acquiring resistance to environmental stresses. Overexpression of either *CBF3* or *ABF3* gene in *Arabidopsis* causes growth inhibition under normal growth condition. Interestingly, transgenic rice overexpressing the *Arabidopsis CBF3* gene exhibits enhanced resistance to drought and high salinity without causing growth defects and phenotypic alterations [176]. It may be due to mismatching of the targets in the heterologous organisms and/or differential transcriptional activation strengths of the transcription factor in *Arabidopsis* and rice [176].

Information on regulatory mechanisms controlling transcription factor activities certainly provides novel insights into genetic engineering of stress-resistant crops. Overexpression of certain transcription factors does not always lead to expected phenotypic alterations and traits. In many cases, additional modification is required, as observed with the ABFs/AREBs and MTF transcription factors. Overexpression of ABFs/AREBs does not lead to phenotypic alterations unless they are phosphorylated [12]. In addition, MTF should be expressed as a truncated form, which is localized into the nucleus [110].

Generation of knockout mutation in crops is extremely difficult because of large size of genomes and polyploidy. In this view, small peptides (siPEPs) would be of choice to selectively suppress specific transcription factors. A large number of transcription factors act as homodimers. Therefore, overexpression of a small interfering protein (peptide) containing the dimerization domain (DD), but lacking the DNA binding domain, may lead to competitive inhibition of functional homodimer formation by forming nonfunctional heterodimers.

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References

1 Yanagisawa, S. (1998) *J. Plant Res.*, **111**, 363–371.
2 Davidson, E.H. (2001) *Genomic Regulatory Systems: Development and Evolution*, Academic Press, San Diego, CA.
3 Wray, G.A., Hahn, M.W., Abouheif, E., Balhoff, J.P., Pizer, M., Rockman, M.V., and Romano, L.A. (2003) *Mol. Biol. Evol.*, **20**, 1377–1419.
4 Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., and Yu, G. (2000) *Science*, **290**, 2105–2110.
5 Luscombe, N.M., Austin, S.E., Berman, H.M., and Thornton, J.M. (2000) *Genome Biol.*, **1**, 1–37.
6 Chinnusamy, V., Schumaker, K., and Zhu, J.K. (2004) *J. Exp. Bot.*, **55**, 225–236.
7 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) *Annu. Rev. Plant Biol.*, **57**, 781–803.
8 Seki, M., Kamei, A., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2003) *Curr. Opin. Biotechnol.*, **14**, 194–199.
9 Zhu, J.K. (2001) *Curr. Opin. Plant Biol.*, **4**, 401–406.
10 Zhu, J.K. (2002) *Annu. Rev. Plant Biol.*, **53**, 247–273.
11 Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M. (2003) *Curr. Opin. Plant Biol.*, **6**, 410–417.
12 Kang, J.Y., Choi, H.I., Im, M.Y., and Kim, S.Y. (2002) *Plant Cell*, **14**, 343–357.
13 Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003) *Plant Cell*, **15**, 63–78.
14 Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) *Curr. Opin. Plant Biol.*, **3**, 217–223.
15 Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2001) *Plant J.*, **27**, 325–333.
16 Shinozaki, K. and Yamaguchi-Shinozaki, K. (2007) *J. Exp. Bot.*, **58**, 221–227.
17 Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 11632–11637.
18 Seo, P.J., Xiang, F., Qiao, M., Park, J.Y., Lee, Y.N., Kim, S.G., Lee, Y.H., Park, W.J., and Park, C.M. (2009) *Plant Physiol.*, **151**, 275–289.
19 Jung, C., Seo, J.S., Han, S.W., Koo, Y.J., Kim, C.H., Song, S.I., Nahm, B.H., Choi, Y.D., and Cheong, J.J. (2008) *Plant Physiol.*, **146**, 623–635.
20 Cominelli, E., Galbiati, M., Vavasseur, A., Conti, L., Sala, T., Vuylsteke, M., Leonhardt, N., Dellaporta, S.L., and Tonelli, C. (2005) *Curr. Biol.*, **15**, 1196–1200.
21 Liang, Y.K., Dubos, C., Dodd, I.C., Holroyd, G.H., Hetherington, A.M., and Campbell, M.M. (2005) *Curr. Biol.*, **15**, 1201–1206.
22 Ren, X., Chen, Z., Liu, Y., Zhang, H., Zhang, M., Liu, Q., Hong, X., Zhu, J.K., and Gong, Z. (2010) *Plant J.*, **63**, 417–429.
23 Li, W.X., Oono, Y., Zhu, J., He, X.J., Wu, J.M., Iida, K., Lu, X.Y., Cui, X., Jin, H., and Zhu, J.K. (2008) *Plant Cell*, **20**, 2238–2251.
24 Mantovani, R. (1999) *Gene*, **239**, 15–27.
25 Lotan, T., Ohto, M., Yee, K.M., West, M.A., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (1998) *Cell*, **93**, 1195–1205.
26 Nelson, D.E., Repetti, P.P., Adams, T.R., Creelman, R.A., Wu, J., Warner, D.C., Anstrom, D.C., Bensen, R.J., Castiglioni, P.P., Donnarummo, M.G., Hinchey, B.S., Kumimoto, R.W., Maszle, D.R., Canales, R.D., Krolikowski, K.A., Dotson, S.B., Gutterson, N., Ratcliffe, O.J., and Heard, J.E. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 16450–16455.
27 Fujita, M., Fujita, Y., Maruyama, K., Seki, M., Hiratsu, K., Ohme-Takagi, M., Tran, L.S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2004) *Plant J.*, **39**, 863–876.
28 Tran, L.S., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K.,

Fujita, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004) *Plant Cell*, **16**, 2481–2498.
29 Tran, L.S., Nakashima, K., Sakuma, Y., Osakabe, Y., Qin, F., Simpson, S.D., Maruyama, K., Fujita, Y., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007) *Plant J.*, **49**, 46–63.
30 Apse, M.P., Aharon, G.S., Snedden, W.A., and Blumwald, E. (1999) *Science*, **285**, 1256–1258.
31 Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D., and Shinozaki, K. (1997) *Plant Cell*, **9**, 1859–1868.
32 He, X.J., Mu, R.L., Cao, W.H., Zhang, Z.G., Zhang, J.S., and Chen, S.Y. (2005) *Plant J.*, **44**, 903–916.
33 Jiang, Y. and Deyholos, M.K. (2009) *Plant Mol. Biol.*, **69**, 91–105.
34 Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F., and Zhang, J.Z. (2002) *Plant Physiol.*, **130**, 639–648.
35 Magome, H., Yamaguchi, S., Hanada, A., Kamiya, Y., and Oda, K. (2008) *Plant J.*, **56**, 613–626.
36 Viswanathan, C. and Zhu, J.K. (2002) *Philos. Trans. R. Soc. Lond B Biol. Sci.*, **357**, 877–886.
37 Cook, D., Fowler, S., Fiehn, O., and Thomashow, M.F. (2004) Proc. *Natl. Acad. Sci.* USA, **101**, 15243–15248.
38 Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M., and Thomashow, M.F. (1998) *Plant J.*, **16**, 433–442.
39 Fowler, S. and Thomashow, M.F. (2002) *Plant Cell*, **14**, 1675–1690.
40 Fowler, S.G., Cook, D., and Thomashow, M.F. (2005) *Plant Physiol.*, **137**, 961–968.
41 Chinnusamy, V., Schumaker, K., and Zhu, J.K. (2003) *J. Exp. Bot.*, **55**, 225–236.
42 Agarwal, M., Hao, Y., Kapoor, A., Dong, C.H., Fujii, H., Zheng, X., and Zhu, J.K. (2006) *J. Biol. Chem.*, **281**, 37636–37645.
43 Hiratsu, K., Ohta, M., Matsui, K., and Ohme-Takagi, M. (2002) *FEBS Lett.*, **514**, 351–354.
44 Vogel, J.T., Zarka, D.G., Van Buskirk, H.A., Fowler, S.G., and Thomashow, M.F. (2005) *Plant J.*, **41**, 195–211.
45 Novillo, F., Medina, J., and Salinas, J. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 21002–21007.
46 Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) *Plant Cell Physiol.*, **47**, 141–153.
47 Zhang, X., Fowler, S.G., Cheng, H., Lou, Y., Rhee, S.Y., Stockinger, E.J., and Thomashow, M.F. (2004) *Plant J.*, **39**, 905–919.
48 Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T., and Thomashow, M.F. (2001) *Plant Physiol.*, **127**, 910–917.
49 Campoli, C., Matus-Cádiz, M.A., Pozniak, C.J., Cattivelli, L., and Fowler, D.B. (2009) *Mol. Genet. Genomics*, **282**, 141–152.
50 Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., and Shinozaki, K. (2002) *Plant J.*, **31**, 279–292.
51 Zhu, J., Shi, H., Lee, B.H., Damsz, B., Cheng, S., Stirm, V., Zhu, J.K., Hasegawa, P.M., and Bressan, R.A. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 9873–9878.
52 Kusano, T., Berberich, T., Harada, M., Suzuki, N., and Sugawara, K. (1995) *Mol. Gen. Genet.*, **248**, 507–517.
53 Lu, G., Paul, A.L., McCarty, D.R., and Ferl, R.J. (1996) *Plant Cell*, **8**, 847–857.
54 Nakagawa, H., Ohmiya, K., and Hattori, T. (1996) *Plant J.*, **9**, 217–227.
55 Levit, J. (1980) *Responses of Plants to Environmental Stresses*, 2nd edn, vol. **1**, Academic Press, New York.
56 Kotak, S., Larkindale, J., Lee, U., von Koskull-Döring, P., Vierling, E., and Scharf, K.D. (2007) *Curr. Opin. Plant Biol.*, **10**, 310–316.
57 Hua, J. (2009) *Curr. Opin. Plant Biol.*, **12**, 568–573.
58 Swindell, W.R., Huebner, M., and Weber, A.P. (2007) *BMC Genomics*, **8**, 125.
59 Nover, L., Bharti, K., Döring, P., Mishra, S.K., Ganguli, A., and Scharf, K.D. (2001) *Cell Stress Chaperones*, **6**, 177–189.

60 Baniwal, S.K., Ganguli, A., Kotak, S., Mishra, S.K., Nover, L., Port, M., Scharf, K.D., Tripp, J., Weber, C., Zielinski, D., and von Koskull-Döring, P. (2004) *J. Biosci.*, **29**, 471–487.

61 Peteranderl, R., Rabenstein, M., Shin, Y., Liu, C.W., Wemmer, D.E., King, D.S., and Nelson, H.C. (1999) *Biochem*, **38**, 383559–383569.

62 von Koskull-Döring, P., Scharf, K.D., and Nover, L. (2007) *Trends Plant Sci.*, **12**, 452–457.

63 Mishra, S.K., Tripp, J., Winkelhaus, S., Tschiersch, B., Theres, L., Nover, K., and Scharf, K.D. (2002) *Genes Dev.*, **16**, 1555–1567.

64 Bharti, K., von Koskull-Döring, P., Bharti, S., Kumar, P., Tintschl-Körbitzer, A., Treuter, E., and Nover, L. (2004) *Plant Cell*, **16**, 1521–1535.

65 Czarnecka-Verner, E., Pan, S., Salem, T., and Gurley, W.B. (2004) *Plant Mol. Biol.*, **56**, 57–75.

66 Kotak, S., Port, M., Ganguli, A., Bicker, F., and von Koskull-Döring, P. (2004) *Plant J.*, **39**, 98–112.

67 Busch, W., Wunderlich, M., and Schöffl, F. (2005) *Plant J.*, **41**, 1–14.

68 Schramm, F., Ganguli, A., Kiehlmann, E., Englich, G., Walch, D., and von Koskull-Döring, P. (2006) *Plant Mol. Biol.*, **60**, 759–772.

69 Nishizawa, A., Yabuta, Y., Yoshida, E., Maruta, T., Yoshimura, K., and Shigeoka, S. (2007) *Plant J.*, **48**, 535–547.

70 Sakuma, Y., Maruyama, K., Qin, F., Osakabe, Y., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 18822–18827.

71 Larkindale, J. and Vierling, E. (2008) *Plant Physiol.*, **146**, 748–761.

72 Schramm, F., Larkindale, J., Kiehlmann, E., Ganguli, A., Englich, G., Vierling, E., and von Koskull-Doring, P. (2008) *Plant J.*, **53**, 264–274.

73 Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F., and Shinozaki, K. (2006) *J. Biol. Chem.*, **281**, 5310–5318.

74 Suzuki, N., Bajad, S., Shuman, J., Shulaev, V., and Mittler, R. (2008) *J. Biol. Chem.*, **283**, 9269–9275.

75 Gao, H., Brandizzi, F., Benning, C., and Larkin, R.M. (2008) *Proc. Natl. Acad. Sci. USA*, **105**, 16398–16403.

76 Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., and Harper, J.F. (2002) *Plant Physiol.*, **130**, 2129–2141.

77 Black, D.L. (2003) *Annu. Rev. Biochem.*, **72**, 291–336.

78 Matlin, A.J., Clark, F., and Smith, C.W. (2005) *Nat. Rev. Mol. Cell Biol.*, **6**, 386–398.

79 Pan, Q., Shai, O., Lee, L.J., Frey, B.J., and Blencowe, B.J. (2008) *Nat. Genet.*, **40**, 1413–1415.

80 Campbell, M.A., Haas, B.J., Hamilton, J.H., Mount, S.M., and Buell, C.R. (2006) *BMC Genomics*, **7**, 327–343.

81 Wang, B.B. and Brendel, V. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 7175–7180.

82 Barbazuk, W.B., Fu, Y., and McGinnis, K.M. (2008) *Genome Res.*, **18**, 1381–1392.

83 Lewis, B.P., Green, R.E., and Brenner, S.E. (2003) *Proc. Natl. Acad. Sci. USA*, **7**, 189–192.

84 Maquat, L.E. (2004) *Nat. Rev. Mol. Cell Biol.*, **5**, 89–99.

85 Ner-Gaon, H., Halachmi, R., Savaldi-Goldstein, S., Rubin, E., Ophir, R., and Fluhr, R. (2004) *Plant J.*, **39**, 877–885.

86 Kim, E., Magen, A., and Ast, G. (2007) *Nucleic Acids Res.*, **35**, 125–131.

87 Lareau, L.F., Green, R.E., Bhatnagar, R.S., and Brenner, S.E. (2004) *Curr. Opin. Cell Biol.*, **14**, 273–282.

88 Egawa, C., Kobayashi, F., Ishibashi, M., Nakamura, T., Nakamura, C., and Takumi, S. (2006) *Genes Genet. Syst.*, **81**, 77–91.

89 Li, J., Li, X., Guo, L., Lu, F., Feng, X., He, K., Wei, L., Chen, Z., Qu, L.J., and Gu, H. (2006) *J. Exp. Bot.*, **57**, 1263–1273.

90 Mastrangelo, A.M., Belloni, S., Barilli, S., Reperti, B., Di Fonzo, N., Stanca, A.M., and Cattivelli, L. (2005) *Planta*, **221**, 705–715.

91 Isshiki, M., Tsumoto, A., and Shimamoto, K. (2006) *Plant Cell*, **18**, 146–158.

92 Palusa, S.G., Ali, G.S., and Reddy, A.S.N. (2007) *Plant J.*, **49**, 1091–1107.

93 Lee, B.H., Kapoor, A., Zhu, J., and Zhu, J.K. (2006) *Plant Cell*, **18**, 1736–1749.

94 Brodersen, P. and Voinnet, O. (2006) *Trends Genet.*, **22**, 268–280.

95 Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., and Voinnet, O. (2008) *Science*, **320**, 1185–1190.

96 Lanet, E., Delannoy, E., Sormani, R., Floris, M., Brodersen, P., Crete, P., Voinnet, O., and Robaglia, C. (2009) *Plant Cell*, **21**, 1762–1768.

97 Jones-Rhoades, M.W. and Bartel, D.P. (2004) *Mol. Cell*, **14**, 787–799.

98 Sunkar, R. and Zhu, J.K. (2004) *Plant Cell*, **16**, 2001–2019.

99 Liu, H.H., Tian, X., Li, Y.J., Wu, C.A., and Zheng, C.C. (2008) *RNA*, **14**, 836–843.

100 Zhou, X., Wang, G., Sutoh, K., Zhu, J.K., and Zhang, W. (2008) *Biochim. Biophys. Acta*, **1779**, 780–788.

101 Chiou, T.J. (2007) *Plant Cell Environ.*, **30**, 323–332.

102 Sunkar, R., Chinnusamy, V., Zhu, J., and Zhu, J.K. (2007) *Trends Plant Sci.*, **12**, 301–309.

103 Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D. (2006) *Science*, **312**, 436–439.

104 Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R., and Zhu, J.K. (2005) *Cell*, **123**, 1279–1291.

105 Wang, L. and Wessler, S.R. (2001) *Plant Physiol.*, **125**, 1380–1387.

106 Wang, X.J., Gaasterland, T., and Chua, N.H. (2005) *Genome Biol.*, **6**, R30.

107 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) *Plant Cell*, **10**, 1391–1406.

108 McGonigle, B., Bouhidel, K., and Irish, V.F. (1996) *Genes Dev.*, **10**, 1812–1821.

109 Ryu, H., Kim, K., Cho, H., Park, J., Choe, S., and Hwang, I. (2007) *Plant Cell*, **19**, 2749–2762.

110 Seo, P.J., Kim, S.G., and Park, C.M. (2008) *Trends Plant Sci.*, **13**, 550–556.

111 Kim, S.Y., Kim, S.G., Kim, Y.S., Seo, P.J., Bae, M., Yoon, H.K., and Park, C.M. (2007) *Nucleic Acids Res.*, **35**, 203–213.

112 Seo, P.J., Kim, M.J., Park, J.Y., Kim, S.Y., Jeon, J., Lee, Y.H., Kim, J., and Park, C.M. (2010) *Plant J.*, **61**, 661–671.

113 Kim, S.G., Kim, S.Y., and Park, C.M. (2007) *Planta*, **226**, 647–654.

114 Kim, S.G., Lee, A.K., Yoon, H.K., and Park, C.M. (2008) *Plant J.*, **55**, 77–88.

115 Yoon, H.K., Kim, S.G., Kim, S.Y., and Park, C.M. (2008) *Mol. Cells*, **25**, 438–445.

116 Liu, J.X., Srivastava, R., Che, P., and Howell, S.H. (2007) *Plant Cell*, **19**, 4111–4119.

117 Liu, J.X., Srivastava, R., Che, P., and Howell, S.H. (2007) *Plant J.*, **51**, 897–909.

118 Iwata, Y., Fedoroff, N.V., and Koizumi, N. (2008) *Plant Cell*, **20**, 3107–3121.

119 Vert, G., and Chory, J. (2006) *Nature*, **441**, 96–100.

120 Nakashima, K., Fujita, Y., Kanamori, N., Katagiri, T., Umezawa, T., Kidokoro, S., Maruyama, K., Yoshida, T., Ishiyama, K., Kobayashi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2009) *Plant Cell Physiol.*, **50**, 45–63.

121 Nagata, T., Niyada, E., Fujimoto, N., Nagasaki, Y., Noto, K., Miyanoiri, Y., Murata, J., Hiratsuka, K., and Katahira, M. (2010) *Proteins*, **78**, 3033–3047.

122 Halford, N.G. and Hey, S.J. (2009) *Biochem. J.*, **419**, 247–259.

123 Fujii, H., Verslues, P.E., and Zhu, J.K. (2007) *Plant Cell*, **19**, 485–494.

124 Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 17306–17311.

125 Vlad, F., Rubio, S., Rodrigues, A., Sirichandra, C., Belin, C., Robert, N., Leung, J., Rodriguez, P.L., Laurière, C., and Merlot, S. (2009) *Plant Cell*, **21**, 3170–3184.

126 Hoth, S., Morgante, M., Sanchez, J.P., Hanafey, M.K., Tingey, S.V., and Chua, N.H. (2002) *J. Cell Sci.*, **115**, 4891–4900.

127 Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.Y.,

Cutler, S.R., Sheen, J., Rodriguez, P.L., and Zhu, J.K. (2009) *Nature*, **462**, 660–664.

128 Nishimura, N., Sarkeshik, A., Nito, K., Park, S.Y., Wang, A., Carvalho, P.C., Lee, S., Caddell, D.F., Cutler, S.R., Chory, J., Yates, J.R., and Schroeder, J.I. (2010) *Plant J.*, **61**, 290–299.

129 Santiago, J., Dupeux, F., Round, A., Antoni, R., Park, S.Y., Jamin, M., Cutler, S.R., Rodriguez, P.L., and Márquez, J.A. (2009) *Nature*, **462**, 665–668.

130 Hubbard, K.E., Nishimura, N., Hitomi, K., Getzoff, E.D., and Schroeder, J.I. (2010) *Genes Dev.*, **24**, 1695–1708.

131 Guo, Y., Xiong, L., Song, C.P., Gong, D., Halfter, U., and Zhu, J.K. (2002) *Dev. Cell*, **3**, 233–244.

132 Song, C.P., Agarwal, M., Ohta, M., Guo, Y., Halfter, U., Wang, P., and Zhu, J.K. (2005) *Plant Cell*, **17**, 2384–2396.

133 Choi, H.I., Park, H.J., Park, J.H., Kim, S., Im, M.Y., Seo, H.H., Kim, Y.W., Hwang, I., and Kim, S.Y. (2005) *Plant Physiol.*, **139**, 1750–1761.

134 Zhu, S.Y., Yu, X.C., Wang, X.J., Zhao, R., Li, Y., Fan, R.C., Shang, Y., Du, S.Y., Wang, X.F., Wu, F.Q., Xu, Y.H., Zhang, X.Y., and Zhang, D.P. (2007) *Plant Cell*, **19**, 3019–3036.

135 Zhu, J., Dong, C.H., and Zhu, J.K. (2007) *Curr. Opin. Plant Biol.*, **10**, 290–295.

136 Smalle, J. and Vierstra, R.D. (2004) *Annu. Rev. Plant Biol.*, **55**, 555–590.

137 Vierstra, R.D. (2009) *Nat. Rev. Mol. Cell Biol.*, **10**, 385–397.

138 Stone, S.L., Williams, L.A., Farmer, L.M., Vierstra, R.D., and Callis, J. (2006) *Plant Cell*, **18**, 3415–3428.

139 Zhang, Y., Yang, C., Li, Y., Zheng, N., Chen, H., Zhao, Q., Gao, T., Guo, H., and Xie, Q. (2007) *Plant Cell*, **19**, 1912–1929.

140 Kurup, S., Jones, H.D., and Holdsworth, M.J. (2000) *Plant J.*, **21**, 143–155.

141 Zhang, X., Garreton, V., and Chua, N.H. (2005) *Genes Dev.*, **19**, 1532–1543.

142 Liu, H. and Stone, S.L. (2010) *Plant Cell*, **22**, 2630–2641.

143 Qin, F., Sakuma, Y., Tran, L.S., Maruyama, K., Kidokoro, S., Fujita, Y., Fujita, M., Umezawa, T., Sawano, Y., Miyazono, K., Tanokura, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2008) *Plant Cell*, **20**, 1693–1707.

144 Dong, C.H., Agarwal, M., Zhang, Y., Xie, Q., and Zhu, J.K. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 8281–8286.

145 Miura, K., Jin, J.B., and Hasegawa, P.M. (2007) *Curr. Opin. Plant Biol.*, **10**, 495–502.

146 Miura, K. and Hasegawa, P.M. (2010) *Trends Cell Biol.*, **20**, 223–232.

147 Kurepa, J., Walker, J.M., Smalle, J., Gosink, M.M., Davis, S.J., Durham, T.L., Sung, D.Y., and Vierstra, R.D. (2003) *J. Biol. Chem.*, **278**, 6862–6872.

148 Lois, L.M., Lima, C.D., and Chua, N.H. (2003) *Plant Cell*, **15**, 1347–1359.

149 Yoo, C.Y., Miura, K., Jin, J.B., Lee, J., Park, H.C., Salt, D.E., Yun, D.J., Bressan, R.A., and Hasegawa, P.M. (2006) *Plant Physiol.*, **142**, 1548–1558.

150 Miura, K., Jin, J.B., Lee, J., Yoo, C.Y., Stirm, V., Miura, T., Ashworth, E.N., Bressan, R.A., Yun, D.J., and Hasegawa, P.M. (2007) *Plant Cell*, **19**, 1403–1414.

151 Miura, K., Lee, J., Jin, J.B., Yoo, C.Y., Miura, T., and Hasegawa, P.M. (2009) *Proc. Natl. Acad. Sci. USA*, **106**, 5418–5423.

152 Conti, L., Price, G., O'Donnell, E., Schwessinger, B., Dominy, P., and Sadanandom, A. (2008) *Plant Cell*, **20**, 2894–2908.

153 Vinson, C., Acharya, A., and Taparowsky, E.J. (2006) *Biochim. Biophys. Acta*, **1759**, 4–12.

154 Amoutzias, G.D., Robertson, D.L., Van de Peer, Y., and Oliver, S.G. (2008) *Trends Biochem. Sci.*, **33**, 220–229.

155 Kim, Y.S., Kim, S.G., Lee, M., Lee, I., Park, H.Y., Seo, P.J., Jung, J.H., Kwon, E.J., Suh, S.W., Paek, K.H., and Park, C.M. (2008) *Plant Cell*, **20**, 920–933.

156 Yun, J., Kim, S.G., Hong, S., and Park, C.M. (2008) *Plant Signal Behav.*, **3**, 615–617.

157 Stockinger, E.J., Mao, Y., Regier, M.K., Triezenberg, S.J., and Thomashow, M.F.

(2001) *Nucleic Acids Res.*, **29**, 1524–1533.

158 Vlachonasios, K.E., Thomashow, M.F., and Triezenberg, S.J. (2003) *Plant Cell*, **15**, 626–638.

159 Sridha, S. and Wu, K. (2006) *Plant J.*, **46**, 124–133.

160 Zhu, J., Jeong, J.C., Zhu, Y., Sokolchik, I., Miyazaki, S., Zhu, J.K., Hasegawa, P.M., Bohnert, H.J., Shi, H., Yun, D.J., and Bressan, R.A. (2008) *Proc. Natl. Acad. Sci. USA*, **105**, 4945–4950.

161 Zhang, K., Sridhar, V.V., Zhu, J., Kapoor, A., and Zhu, J.K. (2007) *PLoS One*, **2**, e1210.

162 Manzanero, S., Rutten, T., Kotseruba, V., and Houben, A. (2002) *Chromosome Res.*, **10**, 467–476.

163 Sokol, A., Kwiatkowska, A., Jerzmanowski, A., and Prymakowska-Bosak, M. (2007) *Planta*, **227**, 245–254.

164 Kim, J.M., To, T.K., Ishida, J., Morosawa, T., Kawashima, M., Matsui, A., Toyoda, T., Kimura, H., Shinozaki, K., and Seki, M. (2008) *Plant Cell Physiol.*, **49**, 1580–1588.

165 Kim, J.M., To, T.K., Nishioka, T., and Seki, M. (2010) *Plant Cell Environ.*, **33**, 604–611.

166 Kwon, C.S., Lee, D., Choi, G., and Chung, W.I. (2009) *Plant J.*, **60**, 112–121.

167 Bustin, M. and Reeves, R. (1996) *Prog. Nucleic Acid Res. Mol. Biol.*, **54**, 35–100.

168 Bianchi, M.E. and Agresti, A. (2005) *Curr. Opin. Genet. Dev.*, **15**, 496–506.

169 Lildballe, D.L., Pedersen, D.S., Kalamajka, R., Emmersen, J., Houben, A., and Grasser, K.D. (2008) *J. Mol. Biol.*, **384**, 9–21.

170 Krohn, N.M., Yanagisawa, S., and Grasser, K.D. (2002) *J. Biol. Chem.*, **277**, 32438–32444.

171 Cavalar, M., Möller, C., Offermann, S., Krohn, N.M., Grasser, K.D., and Peterhänsel, C. (2003) *Biochemistry*, **42**, 2149–2157.

172 Kwak, K.J., Kim, J.Y., Kim, Y.O., and Kang, H. (2007) *Plant Cell Physiol.*, **48**, 221–231.

173 Jerzmanowski, A. (2007) *Biochim. Biophys. Acta*, **1769**, 330–345.

174 Saez, A., Rodrigues, A., Santiago, J., Rubio, S., and Rodriguez, P.L. (2008) *Plant Cell*, **20**, 2972–2988.

175 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) *Nat. Biotechnol.*, **17**, 287–291.

176 Oh, S.J., Song, S.I., Kim, Y.S., Jang, H.J., Kim, S.Y., Kim, M., Kim, Y.K., Nahm, B.H., and Kim, J.K. (2005) *Plant Physiol.*, **138**, 341–351.

21
Make Your Best – MYB Transcription Factors for Improving Abiotic Stress Tolerance in Crops

Andrea Pitzschke

Too hot, too dry, too cold, and too bright. Field crops grow and reproduce in a dynamic and unpredictably changing environment. Adverse climate conditions severely impair plant growth and development. In the face of the growing world population and the climate change, humanity cannot take food production for granted. There is an increasing demand for crops with improved stress tolerance and yield. Understanding the molecular mechanisms that mediate stress adaptation and applying this knowledge to engineering stress-resistant crops is therefore a key to ensure world food security.

MYB domain-containing proteins form a family of transcription factors involved in a diversity of stress-related responses in plants. Interfering with the activity of individual family members often correlates with altered stress tolerance. MYB transcription factors have been thoroughly studied and functionally characterized not only in the model plant *Arabidopsis thaliana* but also in other plant species. Heterologous expression approaches and phylogenetic analyses indicate a good degree of functional conservation.

This conservation will facilitate knowledge transfer, and thus tomorrow's farmers may benefit from today's discoveries in *Arabidopsis*. This chapter reviews the role of MYB transcription factors in abiotic stress signaling. While selected examples from *Arabidopsis* are described, emphasis is given to MYB proteins in field crops. The potentials and limitations of using heterologous expression approaches for genetic engineering of crops with improved stress tolerance are discussed. One part is devoted to mechanisms that regulate MYB protein abundance and activity. A list of valuable data resources (transcription factor databases, transcriptome studies) shall provide fast access to detailed information and bioinformatics tools for researchers interested in a particular plant species.

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21.1 Introduction

21.1.1 Abiotic Stress

As sessile organisms, plants have to endure any condition posed by their environment. Environmental stress is estimated to account for 60–70% of crop yield loss [1]. Owing to global warming, abiotic stresses are gaining even more importance as major constraints on worldwide crop production. The most widespread types of abiotic challenges occurring in the field are drought, osmotic, cold, and heat stresses. In addition, other stresses such as those triggered by ozone and ultraviolet (UV) light are a growing problem. Over the past decades, intensive research has been dedicated to disentangle the molecular mechanisms and identify key regulators of stress adaptation.

Plant stress responses have been most intensively studied in the model plant *Arabidopsis thaliana* and to various extents also in other species. Principal stress adaptation strategies such as osmolyte accumulation under high-salt conditions, production of anthocyanins as UV protectants, and the underlying signaling mechanisms, appear to be largely conserved between species.

21.1.2 Abscisic Acid – A Stress Signaling Hormone

Many stress-related signaling pathways are involve in the production of and regulation by abscisic acid (ABA) [2, 3]. The importance of this plant hormone as mediator of a variety of stress adaptation mechanisms is mirrored by the fact that many mutants displaying an altered stress tolerance also show abnormalities in ABA sensitivity or biosynthesis. As regulator of stomatal aperture, ABA directly controls the plant's water balance.

21.1.3 (Dis)Similarities of Stress Responses

Although any kind of environmental challenge requires a specific adaptation response, the molecular events triggered by diverse abiotic stresses significantly overlap. This is, for example, evidenced in studies comparing transcriptome changes upon cold, salt, or heat treatment in *Arabidopsis* [4], as well as upon cold, drought, or salt treatment in chickpea [5]. Accordingly, a number of mutants and transgenic plants exhibit altered tolerance to, for example, salt, cold, and drought [4, 6].

Often, enhanced stress tolerance is accompanied by a poor growth or seed yield. This may be explained by the relatively high energy cost required for maintaining a permanent "stress awareness." For instance, the *Arabidopsis eskimo* mutant not only tolerates drought, cold, and osmotic stress but also produces fewer seeds [6]. Likewise, enhanced stress tolerance conferred by overexpression of a number of

MYB genes in various plant species is reflected by severe dwarfism [7–10]. An increasing understanding of stress-related signaling mechanisms shall pave the way for improving stress tolerance in crops while maintaining normal plant growth under nonstress conditions.

21.2
Signal Transduction and Amplification

21.2.1
Principle of Signaling Pathways

Plant growth under adverse conditions is a challenging task that requires the establishment of effective stress signaling pathways and networks. Stress adaptation follows a general scheme: (1) stress perception; (2) transduction of the "stress signal" to cytoplasmic components, for example, through modification of receptor binding intracellular proteins; (3) transduction of information into the nucleus; and (4) transcriptional reprogramming, followed by synthesis of stress proteins (e.g., HSPs in heat stress), altered enzyme activities, and metabolic changes (e.g., osmoprotectants upon salt stress, anthocyanins as UV protectants, etc.). Up to now, most signaling pathways are far from being known in their entirety.

21.2.2
Protein Signaling Cascades

Cellular signaling is not only a one-to-one transduction from protein to protein but also involves concomitant amplification of the stress information. Such amplification can be achieved through modification of (a homo- or heterogeneous pool of) multiple target proteins by a single molecule of regulatory enzyme. Prominent players in such catalysis-based signal amplification in the early stress response are protein kinases, such as those of the SERK [11], CDPKs [12], and MAPK family [13–15].

21.2.2.1 **MAPK Cascades**

A highly efficient and specific funneling and amplification of perceived stress signals is achieved if regulatory enzymes are linked in a cascade. Here, mitogen-activated protein kinase (MAPK) cascades are prominent examples. MAPK cascades are signaling modules conserved in eukaryotic species. These cascades minimally consist of three types of kinases, encoded by the gene families of MAPK kinase kinases, MAPK kinases, and MAPKs. MAPK modules serve as both signal transduction and signal amplification: an MAPK kinase kinase phosphorylates multiple molecules of its target MAPK kinase, which in turn phosphorylates and thereby activates a pool of MAPK molecules. The active MAPK then passes on the signal through phosphorylation of downstream targets, including transcription factors. Thus, by altering the properties/activities of target transcription factors, MAPK cascades translate signal perception into altered gene expression. Genetic and

biochemical studies implicate MAPK signaling modules both in biotic and abiotic stress responses and in stomata development [13, 15, 16].

21.2.3 Transcription Factors

Transcription factors that accumulate or are stimulated by an upstream kinase or other regulatory protein subsequently further amplify the perceived stress signal. Few molecules of active transcription factor can drive massive accumulation of target gene transcripts whose translation can yield thousands of thousands of molecules of the respective protein. Thus, transcription factors may be considered as "master switches." Often, their activity is tightly controlled, so as to prevent expression of target genes when there is no demand. Many transcription factors accumulate or are activated at a particular development stage or in a stress-dependent manner. Undoubtedly, transcription factors are very attractive proteins for genetic manipulation to generate stress-resistant crops. Plant transcription factors, according to the type of DNA binding domain, are classified into several families. Many families have been shown to be involved in stress responses. Examples of stress-related transcription factors can be found in all main transcription factor families, including bZIP proteins, WRKY, AP2, NAC, C_2H_2 zinc finger, and MYB proteins [17].

While members of the WRKY family are prominent regulators of biotic stress responses, those of the MYB family primarily act in the signaling of abiotic stresses. In the subsequent chapters, focus will be on MYB transcription factors and their role as regulators of the abiotic stress response and on their potential for engineering crops with improved stress tolerance. Table 21.1 provides an overview of all MYB genes discussed in the chapter, including information on expression and stress-related phenotypes of mutants and overexpressing plants.

21.2.3.1 MYB Transcription Factors – Structure and Phylogeny

MYB proteins form one of the largest families of transcription factors in plants [18], characterized by the conserved DNA binding domain, the MYB domain. The name is derived from the first MYB gene identified, the oncogene v-MYB from the avian *my*elo*b*lastosis virus. MYB proteins are found in animals, fungi, and plants. In animals, they control the development of various types of cancer [19]. The MYB DNA binding domain generally consists of up to three imperfect repeats, R1, R2, and R3, each consisting of 51–53 amino acids.

MYB genes in plants form three major subfamilies, depending on the number of repeats: according to a nomenclature suggested by Stracke *et al.* [20], 1-, 2-, and 3R repeat-containing plant MYB proteins are referred to as MYB1R (Myb-related), R2R3, and MYB3R factors. The largest group, R2R3-type MYB proteins, is exclusively found in plants. Members of this group mainly regulate plant-specific processes [20].

The richest pool of plant MYB research data has been generated in *A. thaliana* and has been reviewed previously [21]. The *Arabidopsis* genome encodes for 126 MYB and

Table 21.1 Overview of plant MYB transcription factors discussed in the chapter.

Species	Gene name/ID	Closest homologue (*A. thaliana*, rice)[a)]	Gene expression (+, induced; −, repressed)	Phenotype (+, enhanced; −, reduced tolerance; o/e, overexpression; k.o., knockout)	Comment	References
Arabidopsis	*AtMYB2* At2g47950		Drought +, ABA +	o/e ABA sensitivity +, salt tolerance +	Microarray available. MYC2/MYB2 coordinately activate Rd22. MYB2o/e are dwarfed	[10]
Arabidopsis	*AtMYB4* At4g38620		UV−	k.o. accumulate sinapate esters, UV resistance + o/e UV resistance −	Transcriptional repressor of rate-limiting step in sinapate ester synthesis contains repressor motif	[56]
Arabidopsis	AtMYB15 AT3G23250		ABA, drought, salt +	o/e drought, salt + ABA sensitivity +, ABA synthesis genes +		[27]
Arabidopsis	*AtMYB44* At5g67300		Various abiotic stresses + flagellin +	k.o. drought −, salt −, impaired stomatal closure o/e ABA hypersensitive, drought +, salt +, enhanced stomatal closure	Directly regulated by MAPK-activated bZIP protein VIP1	[22, 29, 66]
Arabidopsis	*AtMYB60* AT1G08810		Drought −	k.o. drought + o/e in lettuce represses anthocyanin synthesis		[26, 59]
Arabidopsis	*AtMYB96* AT5G62470		ABA, drought +, expressed in guard cells	o/e and activation-tagged mutant ABA sensitivity +, drought +; pathogen tolerance +; dwarfs under normal conditions k.o. *Rd22* expression − normal phenotype	ABA/auxin/SA crosstalk	[9, 28]

(*Continued*)

Table 21.1 (*Continued*)

Species	Gene name/ID	Closest homologue (*A. thaliana*, rice)[a)]	Gene expression (+, induced; −, repressed)	Phenotype (+, enhanced; −, reduced tolerance; o/e, overexpression; k.o., knockout)	Comment	References
Arabidopsis	AtMYB108 (AtBOS1) AT3G06490		*B. cinerea* (necrotroph pathogen) +	k.o. necrotroph pathogens + biotroph pathogens − drought, salt, oxidative stress −		[82]
Resurrection plant *Craterostigma plantagineum*	*CpMYB10* AAM43912	AtMYB78 AT5G49620 Os03g0315400	ABA, drought +	o/e in *A. thaliana* salt +, drought +, ABA sensitivity +, normal phenotype under normal conditions	Might regulate its own promoter	[83]
Rice	*OsMYBS3* AAN63154	MYB-related AT5G47390	Cold +	k.o. cold − o/e cold +; otherwise normal phenotype	Microarray exists; role in long-term cold acclimation	[37]
Rice	*OsMYB4* BAA23340	AtMYB5 AT3G13540	Cold +	o/e in *A. thaliana* and apple drought +, cold + dwarfed apple: modified metabolite accumulation	No data on k.o. or o/e in rice	[7, 8]
Rice	OsMYBR3-2 BAD81765	MYB 3R-5 protein AT5G02320	Cold +	o/e cold + praline levels + higher G2/M-specific gene expression	Binds mitosis-specific *cis*-element	[38]
Wheat	*TAMYB1* ABC86569	AtMYB44 At5g67300 Os02g018770	Drought −	ND		[31]
Wheat	*TAMYB2* AAT37168	AtMYB15 At3g23250 Os04g0517100	Drought +	ND		[31]
Wheat	*TaMYB4 TaMYB5*		Drought +	ND		[31]
Wheat	*TaMYB32*		Salt +	ND		[32]

Wheat	*TaMYBsdu1*	AtMYB20 AT1G66230 OS06g0258000	Drought +, salt + stronger salt-induced expression in salt-tolerant genotype			[33]
Soybean	*GmMYB76* ABH02836	AtMYB2 At2g47190 Os03g0315400	Salt +	o/e salt +, freezing +	Homodimer, heterodimer with GmMYB177	[40]
Soybean	*GmMYB92* ABH02844	AtMYB111 AT5G49330 Os05g0429900	Salt +, cold +	o/e normal salt, freezing tolerance	Homodimer	[40]
Soybean	*GmMYB177* ABH02866	MYB-like *A. thaliana* protein AT5G17300 Os06g0728700	Salt +, drought +	o/e salt +, freezing +	Heterodimer with GmMYB76	[40]
Grapevine	*VvMYBF1* ACV81697	AtMYB12 At2g47460 Hypoth rice MYB GI:125543539	Expressed during ripening; correlates with flavonol accumulation	o/e in *A. thaliana* complements flavonol-deficient phenotype of *Atmyb12*	Specific activator of flavonol synthase	[48]
Grapevine	*VvMYBPA1* AB242302 *VvMYBPA2* DQ886419	AtMYB3 At1g22640 AtMYB113 AT1G66370 OsMYB3 Os03g0410000	Expressed in young berries	o/e (transient) proanthocyanidin +	Transcriptome study of o/e available	[53, 54]
Sweet potato	*IbMYB1* AB258985	AtMYB113 AT1G66370 OsMYB3 Os03g0410000	Highly expressed in purple-fleshed sweet potato	o/e in *A. thaliana* or potato anthocyanin levels + (through induction of all structural anthocyanin genes		[55]

(*Continued*)

Table 21.1 (*Continued*)

Species	Gene name/ID	Closest homologue (*A. thaliana*, rice)[a]	Gene expression (+, induced; −, repressed)	Phenotype (+, enhanced; −, reduced tolerance; o/e, overexpression; k.o., knockout)	Comment	References
Strawberry	*FaMYB1* AF401220	AtMYB6 AT4G09460 Hypothetical OsI_35350	Expressed in ripening fruits	o/e in tobacco: flavonol and anthocyanin synthesis -	Contains repressor motif	[47]
Apple	*MdMYB10* ACQ45201	AtMYB113 AT1G66370 BAD04025	Highly expressed in red-fleshed apple. Expression correlates with color development	o/e in *A. thaliana* high-sorbitol tolerance +, anthocyanins +, osmoprotectants +		[45, 57]

a) Closest homologue using NCBI BlastP.

64 MYB-related proteins [22]. Until now, to approximately half of all *Arabidopsis* MYB proteins, a functional role could be ascribed [21]. *Arabidopsis* MYB factors regulate a diversity of processes, including primary and secondary metabolism, cell fate and identity, and development and stress responses. The identification and functional characterization of MYB proteins in crops is on the rise, and a picture of MYB transcription factors as key players in plant stress management is emerging.

21.3
MYB Proteins in the Model – Abiotic Stress Signaling in *Arabidopsis*

21.3.1
AtMYB2: the Pioneer and Its Partner

The involvement of MYB proteins in the plant response to abiotic stress was first reported in 1997 [23]. In *Arabidopsis*, AtMYB2, a MYB-related protein, and AtMYC2, a bHLH transcription factor, function as transcriptional activators in ABA-inducible gene expression under drought stress. Coordinately, they activate the expression of the drought stress marker gene *AtRd22* through direct binding to *AtRd22* promoter elements [10, 23]; AtMYB2 and AtMYC2 are synthesized *after* the accumulation of endogenous ABA, pointing to a role at a late stage in the stress response. Overexpression of these transcription factors in *Arabidopsis* resulted in ABA hypersensitivity and an enhanced tolerance to osmotic stress [10]. Accordingly, several ABA-inducible genes are among the candidate target genes identified by microarray analysis of *AtMYC2/AtMYB2* overexpressing plants [10]. Although *AtMYC2* overexpressing plants under normal conditions are indistinguishable from wild type, *AtMYB2* and *AtMYC2/AtMYB2* overexpression results in growth retardation.

Meanwhile, *Arabidopsis* drought stress-related MYB proteins have been characterized further. Apparently, they are involved in several aspects of the desiccation response, both as tolerance enhancing and as tolerance repressing factors.

21.3.2
Arabidopsis MYB Proteins as Stomatal Regulators

Stomata are the major sites of gas exchange. Adequate stomatal movement is pivotal to allow CO_2 uptake for photosynthesis on the one hand and to restrict excessive water loss on the other hand. Stomatal pore aperture is mediated by turgor-driven volume exchanges of two surrounding guard cells [24]. Both opening and closing of stomata is regulated by the stress hormone ABA and has been reviewed previously [25]. Briefly, ABA-stimulated accumulation of reactive oxygen species induces stomatal closure via activation of plasma membrane calcium channels. Complementarily, ABA inhibits stomatal opening through downregulation of K^+_{in} channels and H^+-ATPases.

The first plant transcription factor reported to regulate stomatal movement was a MYB protein *Arabidopsis* AtMYB60 [26]. AtMYB60 is specifically expressed in guard cells, and its expression decreases during drought. Loss-of-function mutants show constitutive reduction of stomatal opening and an enhanced drought tolerance. Microarray analysis showed only few genes to be differentially expressed in these mutants, and many are associated with stress responses. The stomata-specific gene expression and the fact that lack of functional AtMYB60, at least in the homologous system (*Arabidopsis*) is affecting only stress-associated pathways and not interfering with growth and development point to a highly specific role. Identifying potential AtMYB60 orthologues in other plant species and repressing their expression would be a targeted and promising strategy to engineer stomatal activity to help crops survive desiccation.

At least three more genes, *AtMYB15* [27], *AtMYB44*, and *AtMYB96* [28], are implicated in drought stress response through stomatal regulation in *Arabidopsis*. *AtMYB15* is expressed in stomatal guard cells. Its expression is induced by ABA, drought, or salt treatments. *AtMYB15* overexpressing lines are hypersensitivity to exogenous ABA and display an improved tolerance to drought and salt stresses. ABA-induced induction of ABA biosynthesis, ABA signaling, and ABA-responsive genes is enhanced in these lines.

Overexpression of *AtMYB44* enhances ABA sensitivity in stomatal closure [29]. *AtMYB44* is an immediate stress-responsive gene whose expression is regulated via a MAPK pathway (see Section 21.6.3.2).

AtMYB96 is primarily expressed in the root. An activation-tagged *myb96* overexpressing mutant (MYB96ox) exhibited an enhanced drought resistance, accompanied by a reduction in stomatal opening upon ABA or drought exposure. The opposite was observed in a *myb96* T-DNA insertional knockout mutant [28]. Stomatal densities were not affected in either of these mutant lines, showing that AtMYB96 regulates specifically stomatal opening.

In addition, AtMYB96ox mutants exhibit dwarfed growth with altered leaf shape and reduced lateral roots. Thus, in contrast to *Atmyb60* knockout mutants, enhanced drought resistance in *AtMYB96*ox mutants is gained at the expense of reduced growth. This growth impairment might in addition be explained by the energy cost paid to an alerted state toward biotic stress: *AtMYB96*ox plants have elevated levels of biotic stress genes and are more resistant to infection with the biotrophic pathogen *Pseudomonas syringae* [9].

21.4 Desiccation, Cold, and Osmotic Stress in Crops

21.4.1 Wheat

Also in other plant species, abiotic stress responses are controlled through multiple MYB proteins. In wheat, 203 MYB and 116 MYB-related genes have been

identified [30], and experimental evidence implicates several family members in stress signaling. Aiming at improving wheat harvest yield, the challenge is now to pick the most relevant candidates (see also Section 21.7).

Upon wheat desiccation, *TaMYB2*, *MYB4*, and *MYB5* are induced (treatment with 30% PEG), while another gene, *TaMYB1*, is repressed [31]. Whether the opposite responsiveness of *TaMYB1* versus *TaMYB2*, *TaMYB4*, *TaMYB5* is due to a crosstalk between the four genes is an interesting question. Another salt stress-induced MYB gene in wheat, *TaMYB32*, was isolated and mapped [32]. All five genes mentioned above still await functional characterization. TaMYB2 might be particularly interesting: its closest homologue in *Arabidopsis* is AtMYB15, a drought-inducible gene that is expressed in stomatal guard cells [27] (see above). It will be interesting to see whether TaMYB2 also displays guard cell-specific expression and, more important, whether similar to AtMYB15 [28], TaMYB2 overexpression also confers salt and drought stress tolerance.

A study examining the expression levels of 10 MYB genes in two recombinant inbred wheat lines that have contrasting salt and drought tolerance revealed four genes responding to short-term salt treatment [33]. One of these genes, *TaMYBsdu1*, is upregulated in leaves and roots under long-term drought stress. Moreover, *TaMYBsdu1* expression is more strongly induced upon salt treatment in the tolerant than in the susceptible genotype. *TaMYBsdu1* could be an important regulator in wheat adaptation to both salt and drought stresses [33]. Its closest homologues in rice and *Arabidopsis* are a MYB protein of unknown function and *AtMYB20/AtMYB85*, respectively. Interestingly, *AtMYB85* expression is upregulated and *AtMYB20* expression downregulated by salt and drought stresses [34, 35]. Neither AtMYB20 nor AtMYB85 loss- or gain-of-function mutants/transgenic plants have been analyzed yet.

21.4.2 Rice

Plants growing in temperate zones have developed a strategy, termed cold acclimation, to withstand subfreezing conditions. Cold acclimation involves the orchestrated action of numerous genes whose expression is controlled by a small number of transcription factors directly responding to the temperature stimulus. Substantial research data exist for several MYB genes to be involved in low-temperature signaling in *Arabidopsis* [21]. Also, in crops evidence for a role of MYB genes in cold adaptation is growing.

Cold is one if not *the* limiting factor in rice production. Particularly at the seedling stage, rice is very sensitive to chilling. Once again, MYB proteins are playing at the front in stress management.

OsMYBS3 is a single MYB domain-containing protein that had been previously implicated in sugar signaling in rice [36]. Sound evidence exists for an additional role of this protein as mediator of cold adaptation [37]: *OsMYBS3* expression is induced by cold. Loss-of-function results in a cold-hypersensitive phenotype, while transgenic rice constitutively overexpressing *OsMYBS3* is more cold tolerant (4 °C for at least 1 week). Thus, *OsMYBS3* appears to be sufficient and necessary for cold tolerance

in rice. Transcriptome profiling of loss- and gain-of-function plants revealed putative OsMYBS3 target genes, including various stress-responsive genes. Interestingly, OsMYBS3 was found to repress the well-known DREB1/CBF-dependent cold signaling pathway. The different kinetics of cold-induced DREB1 (rapid) and OsMYBS3 (slow) expression suggests that distinct pathways act sequentially and complementarily for adapting short- and long-term cold stress in rice [37]. The closest *OsMYBS3* homologue in *Arabidopsis*, At5g47390, is also induced by salt, as well as by ABA and CdCl2 [22], indicating a functional similarity. More important, the enhanced cold tolerance of *OsMYBS3* overexpressing plants is *not* accompanied by yield penalty in normal field conditions [37], making *OsMYBS3* particularly attractive for improving cold tolerance in other crops (Table 21.3).

Equally promising is another cold-inducible *MYB* gene from rice, *OsMYB4*. Constitutive overexpression of *OsMYB4* in *Arabidopsis* conferred enhanced tolerance to several abiotic stresses [8], suggesting that the function is evolutionarily conserved. In fact, it was found later that ectopic expression of *OsMYB4* also improves drought and cold resistance in apple [7]. Despite its promising potential for genetic engineering of other stress-resistant crops, *OsMYB4* will have to be expressed in a more controllable manner (see Section 21.8): both *Arabidopsis* and apple plants constitutively expressing *OsMYB4* showed reduced growth under normal conditions [7, 8]. So far, no studies on *OsMYB4* overexpressing or mutant plants in the homologous system, that is, rice, have been reported.

The rice MYB protein OsMYB3R-2 has also been implicated in the cold response [38]. OsMYB3R-2 specifically binds a mitosis-specific activator *cis*-element, found in the promoters of cyclin-genes. Upon cold treatment, transgenic rice plants overexpressing OsMYB3R-2 have higher transcript levels of several G2/M phase-specific genes. Moreover, these plants display an enhanced cold tolerance, accompanied with elevated levels of the osmoprotectant proline. Therefore, OsMYB3R-2 was proposed to mediate a cold resistance mechanism through regulation of the cell cycle [38].

21.4.2.1 Thirty-Seven Priority Candidates for Cold Signaling in Rice

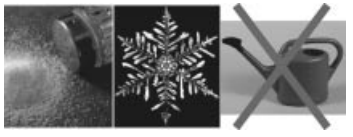
The above examples might only be the tip of the iceberg in MYB-regulated rice chilling response. A genome-wide, physiological and whole-plant-level analysis reported by Yun *et al.* [39] provides a more holistic view of the chilling stress response mechanism in rice.

Within a 96-h duration at which rice seedlings were exposed to 10 °C, 8668 genes showed altered expression. The majority of chilling-induced transcription factors were activated during the initial 24 h. Among these potential main determinants in orchestrating the rice cold response are 37 members of the MYB gene family.

21.4.3 Soybean

In soybean, there are at least 156 MYB genes [40]. For 43 of these, altered expression in response to treatment with ABA, salt, drought, and cold stress was observed. Three candidates, GmMYB76, GmMYB92, and GmMYB177, were studied in more detail.

Table 21.2 (a) Transcriptional profiling studies on abiotic stress responses in crops and (b) useful bioinformatic tools and databases on transcription factors in *Arabidopsis* and field crops.

Species	Description of study	Link/comments	Reference
(a)			
Banana	Heterologous oligonucleotide microarray of drought 	http://www.biomedcentral.com/content/supplementary/1471-2164-10-436-s1.zip	[75]
Chickpea	Microarray of osmotic, cold, drought response 		[5]
M. truncatula	Transcriptome salt-treated roots 	http://bioinformatics.cau.edu.cn/MtED/.	[70]
Rice	Microarray of chilling response 	Contains 37 differentially expressed *MYB* genes	[39]

(*Continued*)

Table 21.2 (*Continued*)

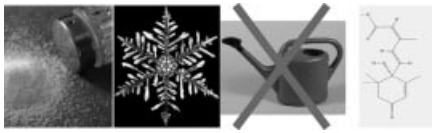
Species	Description of study	Link/comments	Reference
Soybean	Salt, cold, drought, ABA response of *MYB* genes 	http://www.nature.com/cr/journal/v18/n10/extref/cr2008280x2.pdf of 156 MYB genes, 43 are stress responsive	[40]
Sunflower	Salt, cold response 	http://www.sunflowernsa.com/stats/table	[76]
(b)			
Arabidopsis	Database on stress-induced transcription factors	http://caps.ncbs.res.in/stifdb	[84]
Rice	Gene ontology, expression, phylogeny	http://drtf.cbi.pku.edu.cn	[85]
Wheat	Database including 203 MYB proteins affymetrix ID, size, spatial expression	http://wwwappli.nantes.inra.fr:8180/wDBFT/	[30]
Soybean, *L. japonicus*, alfalfa	LegumeTFDB, a database integrating a diversity of information for transcription factors	http://legumetfdb.psc.riken.jp/	[78]
Several species	OstreoTFDB, ChlamyTFDB, ArabTFDB, PoplarTFDB, and RiceTFDB	http://plntfdb.bio.uni-potsdam.de	[86]
	Search tool for putative orthologues	http://pogs.uoregon.edu/	

Table 21.3 Highly attractive MYB genes for genetic engineering, also in heterologous plant species. Research data so far indicate that tolerance is *not* at the expense of growth, development, or seed yield.

Strategy	Reported effect	Studied so far in species
OsMYB3 overexpression	Cold tolerance	Rice
Snapdragon *MYB/MYC* overexpression	Anticancer effect due to anthocyanin accumulation in fruit	Tomato
IbMYB1 overexpression	Potential health-promoting effect due to anthocyanin accumulation	Sweet potato
AtMYB60 inhibition	Drought tolerance, anthocyanin synthesis	*A. thaliana*, lettuce
GmMYB76 or *GmMYB177* overexpression	Salt and freezing tolerance	*A. thaliana*

They exhibit different dimerization characteristics and DNA binding preferences. Overexpression of *GmMYB76* or *GmMYB177*, but not *GmMYB92*, in *Arabidopsis* resulted in an improved salt and freezing tolerance [40]. Together, this study suggests a nonredundant function for these three MYB genes in the plant stress response. The remaining 40 stress-responsive *MYB* genes are further attractive candidates for stress tolerance improvement through genetic engineering.

21.5 Colorful MYB Proteins and Their Merits

Excessive UV radiation triggers the production of reactive oxygen species, followed by cell death. Many plant species produce flavonols and anthocyanins as effective UV protectants. Pigmented anthocyanin compounds also play an important reproductive role as attractants for pollinating insects. On the other hand, accumulation of anthocyanins can prevent plants from excessive herbivory insect attack [41].

For the human diet, the health-promoting effect of anthocyanins is of great interest. Flavonols/anthocyanins offer protection against certain cancers, cardiovascular disease, and age-related degenerative diseases; they have anti-inflammatory activity, promote visual acuity, and hinder obesity and diabetes [42].

The identification and characterization of key regulators in flavonol production in crops may therefore speed up the development of plants with enhanced UV tolerance, improved pollination, and elevated nutritional/health value.

Anthocyanin biosynthesis is controlled by a distinct clade of R2R3 MYB transcription factors [43]. In *Arabidopsis*, a number of MYB factors are involved in the regulation of the phenylpropanoid pathway, mediating anthocyanin and flavonol biosynthesis [21]. This pathway appears to be also regulated by multiple MYB factors in other plant species, including, for instance, apple [44–46], strawberry [47], grapevine [48], persimmon fruit [49], tomato [50], and sweet potato [51].

21.5.1
MYB Proteins for the Human Health

In an impressive scientific report [42], the huge potential of MYB proteins for the engineering of crops with health-promoting activity was demonstrated: ectopic expression of a bHLH and a MYB transcription factor, which had earlier been found to interact to induce anthocyanin biosynthesis in snapdragon [52], in tomato gave rise to fruits with substantially elevated anthocyanin levels. The anthocyanin production in these dark-colored transgenic tomato fruits correlated with a massive increase in antioxidant capacity. Strikingly, cancer-susceptible mice, which were fed with these fruits, attained a longer life span [42]. MYB proteins may therefore open novel approaches for human medicine. Particularly in countries with limited access to a balanced and healthy diet, anthocyanin-accumulating crops may provide a solution.

21.5.2
Anthocyanin Production in Grapevine

For winemakers, sun can be both a blessing and a curse. Quality and quantity of sun is a major determinant in flavor development in ripening wine berries. However, high light and the associated UV radiation are critical challenges for wine plants. A number of wine MYB transcription factors have been identified and characterized to various extents: *VvMYBF1* controls flavonol synthesis through activation of the flavonol synthase gene *VvFLS*. Overexpression of *VvMYBF1* in *Arabidopsis* complements the flavonol-deficient phenotype of *Atmyb12*. This finding highlights the strong conservation and interspecies compatibility of plant MYB proteins [48].

VvMYBPA1 was the first transcription factor found to be involved in the regulation of protocyanin production during seed development in grapevine [53]. Later on, a second gene with high sequence similarity, *VvMYBPA2*, was identified. *VvMYBPA2* is expressed in berries and leaves. The proanthocyanidin profiles in grapevine hairy roots ectopically expressing *VvMYBPA1* or *VvMYBPA2* were altered. Transcriptomic studies of transformed grapevine organs ectopically expressing *VvMYBPA1* or *VvMYBPA2* revealed putative target genes, including several enzymes of the flavonoid pathway [54]. Considering the UV-protective role of anthocyanins and the high light conditions often encountered in vineyards, it will be a worthy experiment to assess the UV tolerance in *VvMYBPA1/2* overexpressing organs or plants.

21.5.3
A Red and Rich Sweet Potato

Purple-fleshed sweet potatoes owe their color – at least partially – to the activity of a MYB transcription factor, IbMYB1 [51]. Transcript levels of this gene are particularly high in the roots of the purple-fleshed variety. *IbMYB* overexpression leads to a drastic

increase in anthocyanin levels through induction of all structural anthocyanin genes, both in sweet potato and in heterologous plant species (*Arabidopsis*). IbMYB1 might therefore be applicable to the production of anthocyanins as nutritive value [55], but it might also serve as a UV protectant in other plant species.

21.5.4
Negative Control of Anthocyanin Production

The above-described proteins, along with a number of other MYB factors from diverse species [43], are all positive regulators of the phenylpropanoid pathway. In contrast, *Arabidopsis AtMYB4* acts as a repressor by inhibiting the expression of a rate-limiting enzyme in this pathway. Accordingly, *Atmyb4* mutants accumulate sinapate esters (major soluble phenylpropanoid metabolites) and exhibit an enhanced UV tolerance, while overexpression – in *Arabidopsis* and tobacco – diminishes sinapate ester levels and results in UV hypersensitivity. The transcriptional repressing effect of AtMYB4 is conferred by a repressor domain located in the C-terminus of the protein [56].

Similar motifs are found in and apparently confer a repressing effect on a small number of other MYB proteins. FaMYB1, which had been isolated from ripening strawberry, represses transcription of anthocyanin-related genes [47]. Heterologous overexpression of FaMYB1 in tobacco represses flavonol and anthocyanin synthesis [47]. Thus, the putative repressor motif contained in FaMYB1 is apparently also functional in the (distant) heterologous species.

21.5.5
Anthocyanins, UV Protection, and the Crosstalk with Other Stressors

Apart from their role as major contributors to UV protection and as attractants for pollinating insects, phenylpropanoid-regulating MYB factors might have a huge yet mainly undiscovered potential; a recent study on the involvement of MdMYB10, a regulator of anthocyanin production in apple fruit [45], in osmotic stress protection is promising: overexpression of *MdMYB10* in *Arabidopsis* resulted in improved growth under high-sorbitol conditions. Accordingly, levels of anthocyanins and osmoprotectants were elevated in these plants [57]. A possible two-sided regulation of both phenylpropanoid pathway and seemingly rather distant stress responses by a single MYB factor is indicated by *Arabidopsis* AtMYB60. The gene is repressed upon UV radiation (www.genevestigator.ethz.ch [58]). *myb60* mutants are more drought tolerant, which is due to a constitutive reduction in stomatal opening (see above section) [26]. Interestingly, when overexpressed in lettuce, AtMYB60 acts as transcriptional repressor of anthocyanin biosynthesis [59]. Recently, also enhanced anthocyanin levels have been reported for AtMYB96 activation-tagged *Arabidopsis* mutants, which are more tolerant to drought [9] and pathogen infection [28]. So far, the salt/drought/temperature tolerance of mutants and transgenic plants with altered content or activity of MYB transcription factors involved in phenylpropanoid biosynthesis has hardly been tested. This is worthy of future study.

21.6
Regulating the Regulators

Members of the MYB factor family not only take an active part in gene regulation, but many are also subject to transcriptional regulation themselves (summarized in Table 21.1). In addition, MYB transcription factors are controlled at the posttranscriptional and posttranslational levels. This is indicative of a sophisticated network fine-tuning MYB-mediated cellular effects. Under acute conditions, plants must be able to quickly prioritize stress responses at the expense of growth. Posttranslational regulation of MYB proteins may serve the immediate response, while their stress-induced transcriptional accumulation is likely to mediate long-term stress adaptation. The major mechanisms controlling MYB expression and activity are described next.

21.6.1
Transcriptional Regulation of MYB Genes

Many MYB factor-encoding genes are expressed in a stimulus-dependent manner. As exemplified in the previous sections, transcript accumulation of individual MYB genes under certain stress conditions has often (successfully) been taken as indicative of the process controlled by the respective proteins. Some MYB transcript levels are altered in response to a diversity of small selection of stimuli or to one type of stress exclusively (www.genevestigator.ethz.ch). A comprehensive survey of transcript abundance of *Arabidopsis MYB* genes in response to various hormone and abiotic stress treatments, as well as a phylogenetic comparison with rice, has highlighted the complex regulation of this gene family in both species [22]. An overview of MYB genes and their response to abiotic stresses can be found in Table 21.1. Transcriptome profiling of plant stress responses and their applicability for MYB research are described in detail in Section 21.7.1.

21.6.2
miRNAs

In *Arabidopsis*, a number of *MYB* genes are targeted by microRNAs. miR159 appears to be a key regulator, controlling at least four AtMYB proteins that are involved in anther and pollen development [21]. Similar posttranscriptional regulatory mechanisms of MYB proteins are likely to exist in other plant species. The identification of miRNAs targeting multiple MYB proteins in crops is a challenging but promising task. Manipulation of miRNAs is a useful tool to repress related, that is, potentially functionally redundant MYB proteins that negatively control stress tolerance. Interesting candidates in this respect are *AtMYB60* and its putative orthologues (see Section 21.3.2).

21.6.3
Posttranslational Regulation of MYB Proteins

Under acute stress conditions, plants need to respond rapidly. Cell damage has to be immediately prevented, even before *de novo* synthesis of signaling components is

accomplished. This can be achieved through posttranslational modification of inactive transcription factors already residing in the cell. Moreover, cells also employ this strategy to quickly inactivate signaling components. Examples of posttranslationally modified proteins can be found in all plant transcription factor families.

Particularly under the aspect of employing transcription factors for genetic engineering of plants, possible posttranslational modifications and their effects on protein activity should be considered. Once again, knowledge about modifications of MYB proteins in (the better-studied) *Arabidopsis* may in some cases be applied to their closest homologues in other species. Major types of posttranslational modification are outlined in the following sections.

21.6.3.1 **SUMOylation**

SUMOylation is a type of posttranslationial modification in which the small ubiquitin-related modifier (SUMO) is reversibly and covalently conjugated to a lysine residue in a substrate protein. This process involves three biochemical steps, SUMO E1 activation, E2 conjugation, and E3 ligation, and is reversed through the action of ubiquitin-like SUMO-specific proteases. SUMOylation can lead to changes in protein–protein interactions, resulting in an altered activity, stability, or localization of substrate proteins [60–62]. Bioinformatic, genetic, and biochemical analyses of *Arabidopsis*, rice, tomato, and *Medicago* indicate that components of the SUMO conjugation and deconjugation systems are conserved in plants [63].

At present, knowledge on SUMOylated proteins in plants is very limited. Putative SUMOylation target motifs (characterized by the minimal consensus motif ΨKXE/D, where Ψ is a large hydrophobic residue; X, any amino acid; E/D, glutamate or aspartate) are found in several plant proteins, including a number of MYB transcription factors. Modification through SUMOylation has been reported for the MYB protein AtLAF1. Disruption of the AtLAF1 SUMO motif resulted in an altered nuclear distribution [64]. However, the functional relevance is still unclear.

A sophisticated mutual regulation between MYB factors and SUMOylation was reported by Miura *et al.* [63]: the *Arabidopsis* MYB-like protein AtPHR1 is a SUMOylation target of the SUMO E3 ligase AtSIZ1, involved in the phosphate starvation response. In turn, SUMOylated AtPHR1 functions as the transcriptional activator of At*SIZ1*.

Another plant SUMO target MYB protein, AtMYB30, has been studied thoroughly *in vitro*, and three SUMOylated lysine residues, located at the C-terminus, have been identified [65]. The experimental system reported by the authors can be applied to test candidate proteins, from any species of interest, for SUMOylation. In this context, it is interesting to note that a few MYB proteins from other plant species display homology to the SUMOylation-site-spanning region of AtMYB30, and at least some of the SUMOylation motifs are conserved (Pitzschke, unpublished).

21.6.3.2 **Phosphorylation**

Phosphorylation is a means of reversible modification that can change the properties of a protein. Protein kinases attach phosphate groups to serine, thereonin, or tyrosine residues of target substrates, while protein phosphatases reverse this process. A fascinating stress-related transcription factor involved in stress and MYB regulation

is the bZIP protein AtVIP1. AtVIP1 has been shown to be a target of the stress-activated kinase AtMPK3 [66]. Upon phosphorylation, AtVIP1 migrates into the nucleus where it activates the expression of stress genes, including AtMYB44. Thus, AtVIP1 directly links MAPK activation to transcriptional reprogramming. Moreover, it further amplifies the stress signal perceived from the MAPK cascade by driving expression of further transcription factors, which in turn modify expression of subsequent target genes [67]. Interestingly, both At*mpk3* [68] and At*myb44* mutants [29] are impaired in ABA-dependent stomatal closure. Whether this defect in At*mpk3* is primarily due to a nonfunctional VIP1–MYB44 pathway is yet to be determined.

Rather than being transcriptionally regulated through MAPKs, some MYB proteins are likely to be directly modified by MAPKs: several MYB proteins are among the candidates identified through a proteomic approach using high-density protein microarrays to determine phosphorylation targets of *Arabidopsis* MAPKs [69]. A MAPK phosphorylation-controlled MYB factor has been reported from pine. PtMYB4 has a potential role in xylem differentiation. Phosphorylation had no effect on (*in vitro*) DNA binding activity of PtMYB4, but it altered its capacity to promote transcription [70].

21.7
Databases and Transcriptome Studies – Resources for MYB Research

Omics-based approaches are promising tools for identifying candidate key regulators in the stress response. A number of links to transcriptome profiles and databases relevant for abiotic stress/MYB research in crops are listed in Table 21.2.

21.7.1
Transcriptomic Profiling of Abiotic Stress Responses in Crops

The characterization of many stress-related plant MYB genes has been preceded by isolation of candidate genes from transcriptome studies. From the vastly increasing information extracted from transcriptomic approaches, combined with large-scale EST sequencing in crops and phylogenetic studies, a global picture is emerging. These rich sources of data, handled in a smart manner, can elucidate stress-specific/cross-species similarities and thus aid the development of more directed approaches toward generating multiple stress-resistant crops. A targeted search for stress signaling components can, for example, be performed by screening published microarrays for genes differentially expressed upon a certain stimulus. This will reveal not only MYB genes as putative regulators, but also their possible downstream targets (i.e., those whose promoters contain MYB binding sites).

A promising and targeted approach to identify MYB factors (and other key components) potentially involved in abiotic stress resistance is the comparison of transcription profiles between non- and stress-exposed plants or between stress-susceptible and -tolerant cultivars.

The latter has, for example, been applied in tomato, where a *MYB* gene is among the transcription factors that show altered expression specifically in drought-tolerant genotypes [71]. Likewise, in wheat *TaMYBsdu1*was found through comparison of expression profiles in wheat cultivars exhibiting contrasting salt and drought tolerance (see above) [33]. Similarly, *IbMYB* was identified as a gene particularly highly expressed in purple-fleshed sweet potato (see above) [55].

Changes in expression profiles in response to stresses have been studied in numerous plant species. Not surprisingly, MYB transcription factors are on the "candidate lists" that arose from such screens. A selection of transcriptome studies is given below, stressing the versatility of such approaches.

By means of microarray analysis of cold-treated rice plants, a number of candidates, including MYB-encoding genes, involved in the chilling response, were identified [39]. Transcript and metabolic profiling data are now also available for common bean, an important legume for human consumption. Phosphorus deficiency is a major limiting factor for nitrogen fixation and is widespread in areas where common bean is grown. From the comparison of P-deficient versus control nodules, a number of candidate genes, including 37 transcription factors, 3 of which are annotated as MYB genes, were identified [72]. In *Arabidopsis*, at least two MYB proteins are involved in the phosphate starvation response (AtPHR1 and *AtMYB62*) [73, 74], once again stressing functional conservation. Given their widespread cultivation, excellent characteristics for the human diet (protein rich!), and the suitability for enriching nitrogen content in soil, leguminous plants are very attractive for research aimed at improving yield/stress tolerance. It is reasonable to assume that MYB proteins act in abiotic stress signaling in probably all legume species. In this context, a database of gene expression profiles of salt-treated roots in *Medicago truncatula* (http://bioinformatics.cau.edu.cn/MtED/) is worth mentioning. This database provides a number of useful bioinformatics tools and is intended to help in selecting gene markers to improve abiotic stress resistance in legumes [70].

A heterologous oligonucleotide microarray approach leads to the identification of drought-induced genes in banana. [75]. Many of these, including MYB genes, displayed homology to genes underlying QTL for drought and cold tolerance in rice. Similar experimental approaches may speed up the discovery of key regulators in abiotic stress signaling in other crop species. First attempts toward microarray studies for the identification of abiotic stress-responsive genes in sunflower have been reported [76].

Despite the merits of microarray studies, one has to be cautious with data interpretation. On the one hand, transcript abundance does not always correlate with protein abundance or activity. On the other hand, most expression studies cover only one or few time points; therefore, genes with transient transcript accumulation might be missed. Even if a stress persists, regulatory proteins (such as MYBs) may not have to be synthesized continuously. Rather, once they have initiated downstream signaling through induction of their target genes, subsequent signaling components "take over." Transient accumulation of MYB transcription factors is also a means of preventing "spillover."

The fine-tuning of MYB-mediated signaling is exemplified by AtMYB4. AtMYB4 negatively regulates the expression of a rate-limiting enzyme in the phenylpropanoid biosynthesis (see above). The repressing effect exerted by AtMYB4 occurs in a dose-dependent manner. Moreover, upon UV exposure, *AtMYB4* expression is down-regulated, indicating that derepression is an important step in acclimation to UV [56].

21.7.2
Transcription Factor Databases

Another milestone in plant stress research was the development of transcription factor databases (TFDB) (see Table 21.2). These have become available for *A. thaliana*, polar rice, *Chlamydomonas reinhardtii*, and *Ostreococcus tauri* [77]. A similar fruitful database and excellent tool providing a more substantial idea on the complexity of the MYB (and other transcription factors) family also exists for wheat [30]. This tool integrates information on 203 MYB and 116 MYB-related genes.

Another example is the database LegumeTFDB, integrating a diversity of information for transcription factors of *Glycine max*, *Leonurus japonicus*, and *M. truncatula*. Hyperlinks to available expression profiles as well as information on *cis*-elements in transcription factor promoters will further aid the identification of stress-related MYB proteins. [78]. For instance, the database can be searched for all TF genes, in a given species, in a given TF family, whose promoters carry a stress-associated *cis*-element(s) of choice. Questioning this database for members of the *G. max MYB* gene family (333 members) that contain MYBR *cis*-element(s) returns 87 hits. In contrast, the larger family of AP2_EREBP transcription factors (405 members) contains proportionally fewer MYBR elements (87/405) (Pitzschke, own observation). The overrepresentation of MYBR elements in promoters of *MYB* genes might be indicative of a sophisticated crosstalk between several MYB members. In addition, autoregulation (a MYB protein regulating its own expression) is also a likely explanation.

21.8
Genetic Engineering, Limitations, Optimizations, Practical Considerations

For the genetic engineering of stress-resistant plants, "simply" overexpressing a transcription factor of interest might often not lead to the anticipated result. Two reasons of failure and means to circumvent these problems are outlined as follows:

1) Overabundance of the transcription factor can have adverse side effects.
2) Posttranslational modification is required for full activity.

1) Most transcription factors carry a nuclear localization signal. Both bioinformatic and *in vivo* studies document that the majority indeed localize to the nucleus, that is, the subcellular site of action. Overexpression of a transcription factor of interest that constitutively localizes to the nucleus and that does not

require posttranslational modification or additional (limiting) transcription factors for reaching full activity might result in hyperactivation of stress genes to an undesirable extent, particularly if growth retardation or developmental defects are the price of enhanced stress resistance conferred by constitutive expression of stress genes. This is exemplified by the dwarfed phenotype of *Arabidopsis AtMYB96* overexpressing plants [28]. Moreover, in several cases attempts to generate transgenic plants constitutively overexpressing *MYB* genes were unsuccessful, possibly due to the toxicity of excessive amounts of the protein (Pitzschke, unpublished) [65].

Here, a more controlled approach might be the method of choice. To minimize negative effects on plant growth due to the use of a constitutive promoter, the use of stress-inducible promoters (*rd29A*, *COR15A*) to drive ectopic expression of transcription factors was previously reported [79, 80]. Alternatively, a chemical-inducible, ecdysone receptor-based system suitable for field applications [81] may be used.

2) In some cases, transcription factors need to be posttranslationally modified to achieve full activity. Such modification can alter the stability, localization, dimerization properties, DNA binding activity, or *cis*-motif preference (see Section 21.6.3). If the site and type of modification is known, protein variants carrying single amino acid exchanges (e.g., mimicking a constitutively phosphorylated state of a transcription factor) should be considered. This strategy has been successfully pursued for PtMYB4 from pine [70] and the MYB44-regulating bZIP protein VIP1 [66]).

21.9 Outlook

The high functional conservation of MYB transcription factors across species will accelerate the process of engineering crops with improved stress tolerance. As many studies have shown, overexpression in heterologous plant species can indeed produce the anticipated stress phenotype.

However, interfering with MYB transcription factor activity has its limitations that one should be aware of: enhanced tolerance to one type of stress might be associated with a reduced tolerance to other stress types. For instance, *Arabidopsis* mutants lacking a functional MYB gene, AtBOS1 (AtMYB108), are more tolerant to infection by a nectrotrophic pathogen (*Botryotinia cinerea*), but have a reduced resistance to drought, salt, and oxidative stress. [82]. Therefore, in-depth studies of a range of stress responses will have to be carried out before engineered plants are ready for the field.

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References

1 Boyer, J.S. (1982) *Science*, **218**, 443.
2 Tuteja, N. (2007) *Plant Signal. Behav.*, **2**, 135.
3 Wasilewska, A., Vlad, F., Sirichandra, C., Redko, Y., Jammes, F., Valon, C., Frei dit Frey, N., and Leung, J. (2008) *Mol. Plant*, **1**, 198.
4 Kant, P., Gordon, M., Kant, S., Zolla, G., Davydov, O., Heimer, Y.M., Chalifa-Caspi, V., Shaked, R., and Barak, S. (2008) *Plant Cell Environ.*, **31**, 697.
5 Mantri, N.L., Ford, R., Coram, T.E., and Pang, E.C. (2007) *BMC Genomics*, **8**, 303.
6 Bouchabke-Coussa, O., Quashie, M.-L., Seoane-Redondo, J., Fortabat, M.-N., Gery, C., Yu, A., Linderme, D., Trouverie, J., Granier, F., Teoule, E., and Durand-Tardif, M. (2008) *BMC Plant Biol.*, **8**, 125.
7 Pasquali, G., Biricolti, S., Locatelli, F., Baldoni, E., and Mattana, M. (2008) *Plant Cell Rep.*, **27**, 1677.
8 Vannini, C., Locatelli, F., Bracale, M., Magnani, E., Marsoni, M., Osnato, M., Mattana, M., Baldoni, E., and Coraggio, I. (2004) *Plant J.*, **37**, 115.
9 Seo, P.J. and Park, C.M. (2010) *New Phytol.*, **186**, 471.
10 Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003) *Plant Cell*, **15**, 63.
11 Santos, M.O. and Aragao, F.J. (2009) *Plant Signal. Behav.*, **4**, 1111.
12 Li, A., Wang, X., Leseberg, C.H., Jia, J., and Mao, L. (2008) *Plant Signal. Behav.*, **3**, 654.
13 Rodriguez, M.C., Petersen, M., and Mundy, J. (2010) *Annu. Rev. Plant Biol.*, **61**, 621.
14 Nakagami, H., Pitzschke, A., and Hirt, H. (2005) *Trends Plant Sci.*, **10**, 339.
15 Colcombet, J. and Hirt, H. (2008) *Biochem. J.*, **413**, 217.
16 Pitzschke, A., Schikora, A., and Hirt, H. (2009) *Curr. Opin. Plant Biol.*, **12**, 421.
17 Singh, K.B., Foley, R.C., and Oñate-Sánchez, L. (2002) *Curr. Opin. Plant Biol.*, **5**, 430.
18 Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., and Yu, G. (2000) *Science*, **290**, 2105.
19 Ramsay, R.G. and Gonda, T.J. (2008) *Nat. Rev. Cancer*, **8**, 523.
20 Stracke, R., Werber, M., and Weisshaar, B. (2001) *Curr. Opin. Plant Biol.*, **4**, 447.
21 Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., and Lepiniec, L. (2010) *Trends Plant Sci.*, **15**, 573.
22 Yanhui, C., Xiaoyuan, Y., Kun, H., Meihua, L., Jigang, L., Zhaofeng, G., Zhiqiang, L., Yunfei, Z., Xiaoxiao, W., Xiaoming, Q., Yunping, S., Li, Z., Xiaohui, D., Jingchu, L., Xing-Wang, D., Zhangliang, C., Hongya, G., and Li-Jia, Q. (2006) *Plant Mol. Biol.*, **60**, 107.
23 Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D., and Shinozaki, K. (1997) *Plant Cell*, **9**, 1859.
24 MacRobbie, E.A. (1998) *Philos. Trans. R. Soc. Lond. B*, **353**, 1475.
25 Kim, T.H., Bohmer, M., Hu, H., Nishimura, N., and Schroeder, J.I. (2010) *Annu. Rev. Plant Biol.*, **61**, 561.
26 Cominelli, E., Galbiati, M., Vavasseur, A., Conti, L., Sala, T., Vuylsteke, M., Leonhardt, N., Dellaporta, S.L., and Tonelli, C. (2005) *Curr. Biol.*, **15**, 1196.
27 Ding, Z., Li, S., An, X., Liu, X., Qin, H., and Wang, D. (2009) *J. Genet. Genomics*, **36**, 17.
28 Seo, P.J., Xiang, F., Qiao, M., Park, J.-Y., Lee, Y.N., Kim, S.-G., Lee, Y.-H., Park, W.J., and Park, C.-M. (2009) *Plant Physiol.*, **151**, 275.
29 Jung, C., Seo, J.S., Han, S.W., Koo, Y.J., Kim, C.H., Song, S.I., Nahm, B.H., Choi, Y.D., and Cheong, J.J. (2008) *Plant Physiol.*, **146**, 623.
30 Romeuf, I., Tessier, D., Dardevet, M., Branlard, G., Charmet, G., and Ravel, C. (2010) *BMC Genomics*, **11**, 185.
31 Chen, R., Ni, Z., Nie, X., Qin, Y., Dong, G., and Sun, Q. (2005) *Plant Sci.*, **169**, 1146.
32 Zhang, L.-C., Zhao, G.-Y., Jia, J.-Z., and Kong, X.-Y. (2009) *Acta Agron. Sin.*, **35**, 1181.

33 Rahaie, M., Xue, G.P., Naghavi, M.R., Alizadeh, H., and Schenk, P.M. (2010) *Plant Cell Rep.*, **29**, 835.

34 Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., and Provart, N.J. (2007) *PLoS One*, **2**, e718.

35 Zeller, G., Henz, S.R., Widmer, C.K., Sachsenberg, T., Ratsch, G., Weigel, D., and Laubinger, S. (2009) *Plant J.*, **58**, 1068.

36 Lu, C.-A., Ho, T.-H.D., Ho, S.-L., and Yu, S.-M. (2002) *Plant Cell*, **14**, 1963.

37 Su, C.-F., Wang, Y.-C., Hsieh, T.-H., Lu, C.-A., Tseng, T.-H., and Yu, S.-M. (2010) *Plant Physiol.*, **153**, 145.

38 Ma, Q., Dai, X., Xu, Y., Guo, J., Liu, Y., Chen, N., Xiao, J., Zhang, D., Xu, Z., Zhang, X., and Chong, K. (2009) *Plant Physiol.*, **150**, 244.

39 Yun, K.Y., Park, M.R., Mohanty, B., Herath, V., Xu, F., Mauleon, R., Wijaya, E., Bajic, V.B., Bruskiewich, R., and de Los Reyes, B.G. (2010) *BMC Plant Biol.*, **10**, 16.

40 Liao, Y., Zou, H.F., Wang, H.W., Zhang, W.K., Ma, B., Zhang, J.S., and Chen, S.Y. (2008) *Cell Res.*, **18**, 1047.

41 Karageorgou, P., and Manetas, Y. (2006) *Tree Physiol.*, **26**, 613.

42 Butelli, E., Titta, L., Giorgio, M., Mock, H.P., Matros, A., Peterek, S., Schijlen, E.G., Hall, R.D., Bovy, A.G., Luo, J., and Martin, C. (2008) *Nat. Biotechnol.*, **26**, 1301.

43 Allan, A.C., Hellens, R.P., and Laing, W.A. (2008) *Trends Plant Sci.*, **13**, 99.

44 Ban, Y., Honda, C., Hatsuyama, Y., Igarashi, M., Bessho, H., and Moriguchi, T. (2007) *Plant Cell Physiol.*, **48**, 958.

45 Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Amma, S., and Allan, A.C. (2007) *Plant J.*, **49**, 414.

46 Takos, A.M., Jaffe, F.W., Jacob, S.R., Bogs, J., Robinson, S.P., and Walker, A.R. (2006) *Plant Physiol.*, **142**, 1216.

47 Aharoni, A., De Vos, C.H., Wein, M., Sun, Z., Greco, R., Kroon, A., Mol, J.N., and O'Connell, A.P. (2001) *Plant J.*, **28**, 319.

48 Czemmel, S., Stracke, R., Weisshaar, B., Cordon, N., Harris, N.N., Walker, A.R., Robinson, S.P., and Bogs, J. (2009) *Plant Physiol.*, **151**, 1513.

49 Akagi, T., Ikegami, A., Tsujimoto, T., Kobayashi, S., Sato, A., Kono, A., and Yonemori, K. (2009) *Plant Physiol.*, **151**, 2028.

50 Ballester, A.R., Molthoff, J., de Vos, R., Hekkert, B.L., Orzaez, D., Fernandez-Moreno, J.P., Tripodi, P., Grandillo, S., Martin, C., Heldens, J., Ykema, M., Granell, A., and Bovy, A. (2010) *Plant Physiol.*, **152**, 71.

51 Mano, H., Ogasawara, F., Sato, K., Higo, H., and Minobe, Y. (2007) *Plant Physiol.*, **143**, 1252.

52 Schwinn, K., Venail, J., Shang, Y., Mackay, S., Alm, V., Butelli, E., Oyama, R., Bailey, P., Davies, K., and Martin, C. (2006) *Plant Cell*, **18**, 831.

53 Bogs, J., Jaffe, F.W., Takos, A.M., Walker, A.R., and Robinson, S.P. (2007) *Plant Physiol.*, **143**, 1347.

54 Terrier, N., Torregrosa, L., Ageorges, A., Vialet, S., Verries, C., Cheynier, V., and Romieu, C. (2009) *Plant Physiol.*, **149**, 1028.

55 Kim, C.Y., Ahn, Y.O., Kim, S.H., Kim, Y.H., Lee, H.S., Catanach, A.S., Jacobs, J.M., Conner, A.J., and Kwak, S.S. (2010) *Physiol Plant*, **139**, 229.

56 Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., Tonelli, C., Weisshaar, B., and Martin, C. (2000) *Embo J.*, **19**, 6150.

57 Gao, J.J., Zhang, Z., Peng, R.H., Xiong, A.S., Xu, J., Zhu, B., and Yao, Q.H. (2010) *Mol. Biol. Rep.*, **38**, 205.

58 Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004) *Plant Physiol.*, **136**, 2621.

59 Park, J.-S., Kim, J.-B., Cho, K.-J., Cheon, C.-I., Sung, M.-K., Choung, M.-G., and Roh, K.-H. (2008) *Plant Cell Rep.*, **27**, 985.

60 Johnson, E.S. (2004) *Annu. Rev. Biochem.*, **73**, 355.

61 Melchior, F., Schergaut, M., and Pichler, A. (2003) *Trends Biochem. Sci.*, **28**, 612.

62 Schmidt, D. and Muller, S. (2003) *Cell Mol. Life Sci.*, **60**, 2561.

63 Miura, K., Jin, J.B., and Hasegawa, P.M. (2007) *Curr. Opin. Plant Biol.*, **10**, 495.

64 Ballesteros, M.L., Bolle, C., Lois, L.M., Moore, J.M., Vielle-Calzada, J.P., Grossniklaus, U., and Chua, N.H. (2001) *Genes Dev.*, **15**, 2613.

65 Okada, S., Nagabuchi, M., Takamura, Y., Nakagawa, T., Shinmyozu, K., Nakayama, J.-I., and Tanaka, K. (2009) *Plant Cell Physiol.*, **50**, 1049.

66 Djamei, A., Pitzschke, A., Nakagami, H., Rajh, I., and Hirt, H. (2007) *Science*, **318**, 453.

67 Pitzschke, A., Djamei, A., Teige, M., and Hirt, H. (2009) *Proc. Natl. Acad. Sci. USA*, **106**, 18414.

68 Gudesblat, G.E., Iusem, N.D., and Morris, P.C. (2007) *New Phytol.*, **173**, 713.

69 Popescu, S.C., Popescu, G.V., Bachan, S., Zhang, Z., Gerstein, M., Snyder, M., and Dinesh-Kumar, S.P. (2009) *Genes Dev.*, **23**, 80.

70 (a) Li, D., Su, Z., Dong, J., and Wang, T. (2009) *BMC Genomics*, **10**, 517; (b) Morse, A.M., Whetten, R.W., Dubos, C., and Campbell, M.M. (2009) *New Phytol.*, **183**, 1001.

71 Gong, P., Zhang, J., Li, H., Yang, C., Zhang, C., Zhang, X., Khurram, Z., Zhang, Y., Wang, T., Fei, Z., and Ye, Z. (2010) *J. Exp. Bot.*, **61**, 3563.

72 Hernandez, G., Valdes-Lopez, O., Ramirez, M., Goffard, N., Weiller, G., Aparicio-Fabre, R., Fuentes, S.I., Erban, A., Kopka, J., Udvardi, M.K., and Vance, C.P. (2009) *Plant Physiol.*, **151**, 1221.

73 Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A.S., Raghothama, K.G., Baek, D., Koo, Y.D., Jin, J.B., Bressan, R.A., Yun, D.-J., and Hasegawa, P.M. (2005) *Proc. Natl. Acad. Sci. USA*, **102**, 7760.

74 Devaiah, B.N., Madhuvanthi, R., Karthikeyan, A.S., and Raghothama, K.G. (2009) *Mol. Plant*, **2**, 43.

75 Davey, M.W., Graham, N.S., Vanholme, B., Swennen, R., May, S.T., and Keulemans, J. (2009) *BMC Genomics*, **10**, 436.

76 Fernandez, P., Di Rienzo, J., Fernandez, L., Hopp, H.E., Paniego, N., and Heinz, R.A. (2008) *BMC Plant Biol.*, **8**, 11.

77 Riano-Pachon, D.M., Correa, L.G., Trejos-Espinosa, R., and Mueller-Roeber, B. (2008) *Genetics*, **179**, 31.

78 Mochida, K., Yoshida, T., Sakurai, T., Yamaguchi-Shinozaki, K., Shinozaki, K., and Tran, L.S. (2010) *Bioinformatics*, **26**, 290.

79 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) *Nat. Biotechnol.*, **17**, 287.

80 Pellegrineschi, A., Reynolds, M., Pacheco, M., Brito, R.M., Almeraya, R., Yamaguchi-Shinozaki, K., and Hoisington, D. (2004) *Genome*, **47**, 493.

81 Padidam, M., Gore, M., Lu, D.L., and Smirnova, O. (2003) *Transgenic Res.*, **12**, 101.

82 Mengiste, T., Chen, X., Salmeron, J., and Dietrich, R. (2003) *Plant Cell*, **15**, 2551.

83 Villalobos, M.A., Bartels, D., and Iturriaga, G. (2004) *Plant Physiol.*, **135**, 309.

84 Shameer, K., Ambika, S., Varghese, S.M., Karaba, N., Udayakumar, M., and Sowdhamini, R. (2009) *Int. J. Plant Genomics*, **2009**, 583429.

85 Gao, G., Zhong, Y., Guo, A., Zhu, Q., Tang, W., Zheng, W., Gu, X., Wei, L., and Luo, J. (2006) *Bioinformatics*, **22**, 1286.

86 Riano-Pachon, D.M., Ruzicic, S., Dreyer, I., and Mueller-Roeber, B. (2007) *BMC Bioinformatics*, **8**, 42.

22
Transporters and Abiotic Stress Tolerance in Plants

Vandna Rai, Narendra Tuteja, and Teruhiro Takabe

Salt and drought are important factors in reducing the crop yields. Transporters play significant roles for stress tolerance in plants. After completion of gene sequencing of rice and *Arabidopsis*, rapid progresses in plant transporters have been made. Here, recent progresses in the plant transporters such as NHX, HKT, sucrose transporters and amino acid transporters for abiotic stress tolerance are discussed. Recently, whole-genome expression profiling has been done to get the information on the roles of transporters under drought and salt stress conditions. Salt- and/or drought-tolerant and sensitive rice varieties, as well as expression profiling for *Arabidopsis* transporters, were used to obtain the important genes for salt/drought stresses. Recent progress on these approaches has also been discussed.

22.1
Introduction

World population is increasing at an alarming rate to reach about 9 billion by the end of 2050. On the other hand, increase in food productivity is hampered by worsening environmental problems. Salt and drought are major factors in decreasing crop production on irrigated land worldwide. A recent estimate by FAO suggested that around 6% of the world's total land area and 20% of irrigated land are affected by high salinity [1]. In India, 9 million ha of rice area is affected by salinity, leading to a considerable loss of grain yield. Thus, there is an urgent need to develop varieties that can grow well in saline environments to enable the farmers to reap good harvests. However, there are only few instances where salt-tolerant cultivars have been developed. The reason for this is that salt stress is a very complex trait, has many components, and is caused by the coordinated action of multiple stress-responsive genes. Recent progress in genetic approach is remarkable. Analysis of various genomes suggested that 10% of all the proteins functions as transporters, and in *E. coli* 42% of proteins are transporters. About 200 families of

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transporters are reported, among which ABC is the largest family. Here, transcriptome and functional analysis of plant transporters in salt and drought stresses will be described.

22.2 Basic Description of Transporters

Transporters vary in their structure and size from small organic molecules and peptides to multisubunit complexes. Membrane transport proteins are divided into three categories: pump, channel, and secondary transporters. Two modes of transport across membranes are known: one is the simple diffusion where no carriers are involved and the other is carrier-mediated diffusion. There are two modes of mediated diffusion, one is passive transport and other is active transport (Figure 22.1). Passive transport is energy independent and occurs following the concentration gradient as mobile carriers (valinomycin, nigericin, dinitrophenol, etc.), protein translocators (porins, erythrocyte glucose transporter), channel-forming ionophores (gramicidin), voltage-gated channels (Na^+, K^+, and Ca^{2+} channels), ligand-gated channels, and mechanosensitive channels. Another is an active transport that is energy dependent and occurs against concentration gradient. It is the primary active

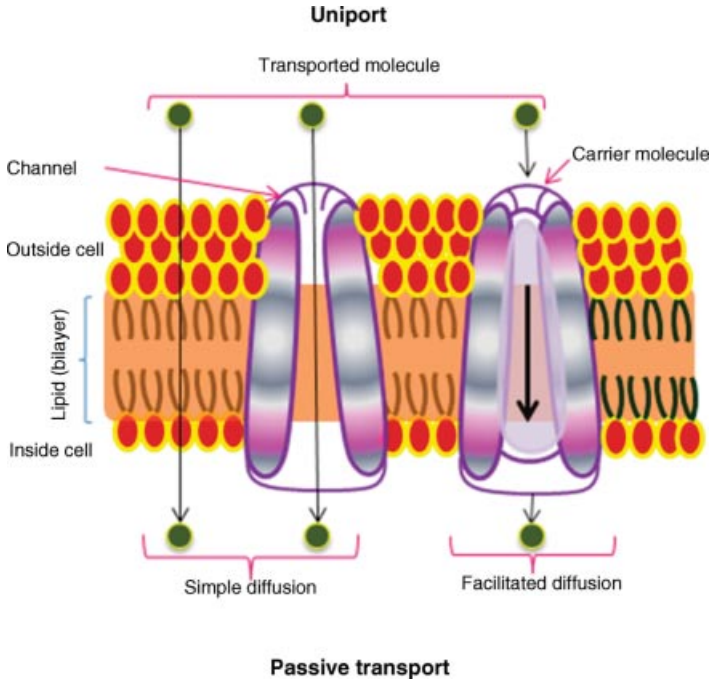

Figure 22.1 Mechanism for passive transport.

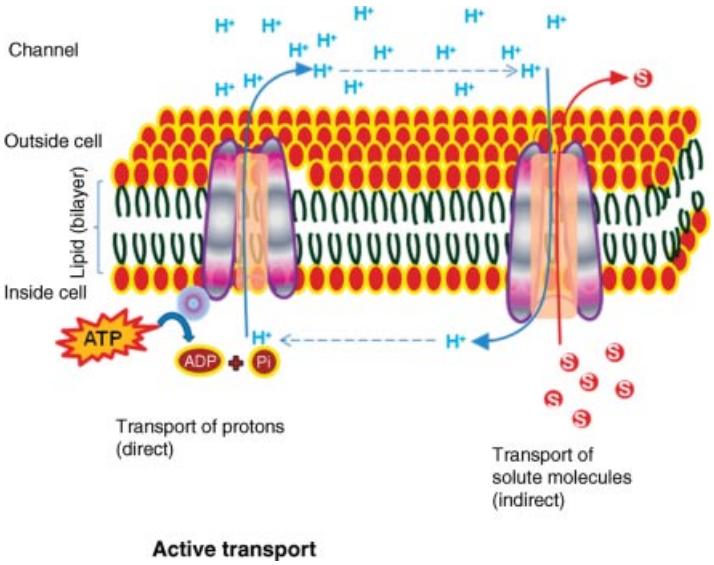

Figure 22.2 Mechanism for energy-dependent transport.

transport that utilizes energy of ATP hydrolysis that includes P-type ATPases (H^+/K^+-ATPases, Ca^{2+}-ATPase), V-ATPases, and F_1F_0-ATPases (Na^+-ATPase, H^+-ATPase) (Figure 22.2). The main roles of the ATP-dependent (pump) proteins include transport of molecules in specific directions independent of the environmental situation and transport of ions to form a concentration gradient (active transport). The secondary transporter system works through concentration gradient dependent on cotransporter molecules (Figure 22.3). More than 100 species of secondary transporter gene families are described [2]. Compared to those in the pump and

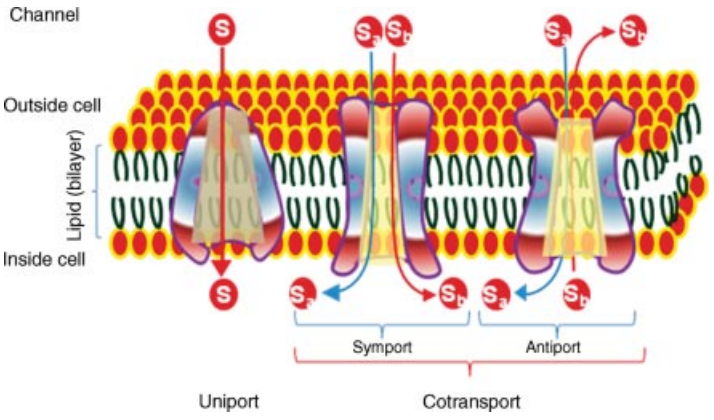

Figure 22.3 Mechanism for secondary transporters.

channel systems, the genes involved in the secondary transporter system are most divergent in plants. In plant system, many cations, anions, metals, and drugs are cotransported with H^+ ions. Only few transporters use Na^+ ions as cotransporter molecules for efflux of ions from the cytoplasm, so plants do not need sodium uptake. For secondary transport, different types are known, such as symporters (two solutes move in same direction, lac permease, Na^+/glucose transporter), antiporters (two solutes move in opposite directions, Na^+/H^+ antiporter), and uniporters (mitochondrial Ca^{2+} transporter). Ion channels have the "gates" in the membranes that help in opening or closing of gates with response to signals such as mechanical or electrical stimulation and ligand binding. Many channel genes for ion homeostasis and signal transduction (CytB and MscS) are found in plants. The MIP (major intrinsic protein) gene family encoding water transport proteins are well developed in plants. MIP proteins are abundant in the plasma membrane (15–20% of total membrane protein) and vacuoles (30–50% of total membrane protein) of plants. Therefore, a high level of water transport is carried out at plant cell membranes.

22.3 Role of Transporters for Salt Tolerance in Plants

22.3.1 Na^+ Transporter

Plant salinity tolerance is a function of Na^+ exclusion, tissue tolerance to Na^+, and osmotic tolerance [3] and depends to a varying extent on each of these three components, even within a single species [4]. Water and ions (primarily Na^+, K^+, and Cl^-) transport is the key to these three mechanisms of salinity tolerance for transport both into and throughout the plant as this transport is highly interconnected with salinity tolerance (Figure 22.4). For saline soils, NaCl is the dominant salt and both Na^+ and Cl^- are metabolically toxic to plants at high concentrations in the cytoplasm.

An extensive study was done on model plant *Arabidopsis* for salinity stress and many genes were found out for salt tolerance that consisted of a group of transporters, including AtNHX1, AtSOS1, and AtHKT1 [5–12]. The *AtNHX1* gene was identified by sequence homology to the *Nhx1* gene in *Saccharomyces cerevisiae*. The AtNHX1 transporter functions in Na^+ sequestration into the vacuole during salinity stress to maintain a high K^+/Na^+ ratio in the cytosol [6, 7]. The salt overly sensitive 1 (*SOS1*) gene, which has been identified in a genetic screen [8], encodes a membrane protein that is homologous to plasma membrane Na^+/H^+ antiporters from bacteria and fungi [13]. Mutant analyses of *sos1* plants have led to a working model of the SOS1 transporter under salinity stress, where SOS1 prevents Na^+ level in xylem sap by unloading/loading of Na^+ into xylem vessels decided by the strength of salinity stress, and influenced Na^+ transport from roots to shoots [13]. SOS1 also functions in direct Na^+ extrusion to outer environment from the root tip where meristematic cells do not have large vacuoles for Na^+ sequestration [13]. Direct Na^+ extrusion by

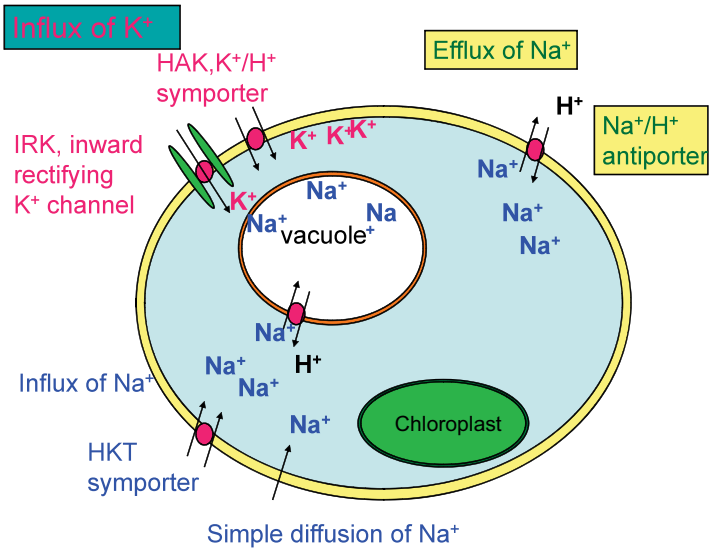

Figure 22.4 Plant cell showing NHX and HKT type of transporters function.

SOS1 from mature epidermal zones of *Arabidopsis* roots was also suggested [14]. *AtHKT1;1* was identified as an *Arabidopsis* homologue of the wheat *TaHKT2;1* [9, 15]. When expressed in heterologous systems such as *S. cerevisiae* and *Xenopus laevis* oocytes, AtHKT1;1 prefers selective Na^+ transport. The disruption of the *AtHKT1;1* gene makes plants hypersensitive to Na^+ resulting in severe leaf chlorosis and Na^+ overaccumulation in shoots under saline conditions [10, 16–19]. For some species such as rice and wheat, the concentrations of Na^+, but not Cl^-, in shoot or root are negatively correlated with salt tolerance [20–23], while the reverse is true for other species. In soybean, leaf Cl^- was negatively correlated with salt tolerance, but there was no significant correlation for leaf Na^+ [24].

22.3.1.1 Uptake of Na^+ to the Root

Na^+ uptake is mediated by Ca^{2+}-sensitive and -insensitive processes. Addition of up to 10 mm Ca^{2+} to the external solution will (generally) reduce the toxic effects of Na^+ [24], probably due to the inhibition of unidirectional Na^+ influx by Ca^{2+}. Ca^{2+}-sensitive Na^+ influx likely occurs through nonselective cation channels (NSCCs) [25]. Many candidates for NSCCs are present, such as cyclic nucleotide-gated channels (CNGCs) [26] and glutamate-activated channels (GLRs), but still the genetic nature of NSCCs is not well known.

22.3.2 K^+ Transporter

Potassium (K^+) is an essential macronutrient that is required for diverse cellular processes, such as osmotic regulation, maintenance of membrane potential, enzyme

activity, protein and starch synthesis, respiration, and photosynthesis. Various studies have indicated that increasing cytosolic K^+ levels relative to Na^+, thus increasing the K^+/Na^+ ratio, is crucial for Na^+ tolerance in plants, and maintaining high K^+/Na^+ ratio in shoots is highly correlated with salinity tolerance in glycophytes [17, 28]. Physiological experiments have shown that the K^+ content in glycophytes can reach 1.5–5% of the dry weight, and the majority of K^+ absorbed in roots are transported to shoots, which is a major limiting factor of the shoot growth and yields of crop plants. Since excessive Na^+ ions inhibit various important cellular processes, many of which are directly correlated with K^+ transport and essential functions of K^+, it is not surprising to see that K^+ alleviates toxic effects of Na^+ and that a high K^+/Na^+ ratio in shoots, especially in leaves, is preferred by glycophytes.

Plant cells utilize K^+ as a major osmotically active solute to maintain turgor and drive irreversible cell expansion and reversible changes in cell volume. Potassium is a major factor in resistance to drought, salinity, and fungal diseases [29]. Plants possess a large number of genes encoding K^+ transporters, including high-affinity transporters and ion channels [10]. The K^+ channels contribute to more than 50% of the nutritional K^+ uptake under most field conditions [30]. *Arabidopsis thaliana* AKT1 (*Arabidopsis* K^+ transporter 1) was reported to be channel mediated that is regulated by voltage (a process known as gating in which the channels open and close), and in this case to favor channel opening at negative voltages that promote net K^+ influx. AKT1 and its relatives are members of the Kv-like (Shaker-like) family of channel proteins that form functional units as tetrameric assemblies around a central pore [31, 32]. Other channels such as SKOR and GORK facilitate K^+ flux outward across the plant plasma membrane. In the root, SKOR is primarily localized to xylem parenchyma cells and is thought to enable the efflux of K^+ from these cells into the xylem for transport to the shoot [33]. GORK contributes to stomatal closure, which is important for plant adaptation to environmental change [34]. Membrane depolarization is required for potassium ion flux through SKOR and GORK. Both SKOR and GORK share the same basic structural features of the inward-rectifying K^+ channels, including AKT1 and KAT1 (K^+ channel of *A. thaliana* 1), although their gating characteristics differ profoundly.

22.3.3 Cl^- Transporters

Mechanisms of Cl^- transport in plants are not well known; however, it is very important to minimize Cl^- toxicity for salt tolerance. Key aspects of Cl^- transport that contribute to salt tolerance in some species include reduced net xylem loading, intracellular compartmentation and greater efflux of Cl^- from roots. Candidate genes for anion transporters are identified that may be contributing to Cl^- movement within plants during salinity [35]. The initial uptake of Cl^- (and Na^+) at the root–soil interface would control the transport of these ions to shoots. NaCl-induced efflux of Cl^- has been observed in several species, for example, in the halophyte *Diplachne fusca* [36], barley [37, 38], sorghum [39], and *Arabidopsis* [40]. Genotypic differences in Cl^- efflux have also been observed. In a recent study, using scanning ion-selective

electrodes, roots of salt-tolerant *Populus euphratica* treated with 100 mm NaCl for 15 days exhibited a significant net efflux of Cl^- at 400–1200 mM from the apex [41]. Under the same conditions, no efflux of Cl^- was observed in the salt-sensitive *Populus popularis* and responses were NaCl specific (and not a result of hyperosmotic stress). The conclusion from this study was that Cl^- (and Na^+) extrusion from *P. euphratica* roots contributed to ion homoeostasis under saline conditions [41].

22.3.3.1 Intercellular Compartmentation of Cl^- is One Way to Cope with High Cl^- Toxicity

Compartmentalization of Cl^- between different cell types in both roots and shoots contributes to salt tolerance in some species. In leaves, Cl^- is preferentially accumulated in the epidermis, reducing Cl^- toxicity in mesophyll cells that are more important for photosynthesis. A salt-tolerant cultivar of barley was more effective in excluding Cl^- from mesophyll cells, compared to a more sensitive barley cultivar [42]. In a study comparing a salt-tolerant barley cultivar with a salt-sensitive durum wheat, Cl^- was preferentially accumulated in the epidermis compared to the mesophyll, but to a similar extent in both species [43]; thus, allocation of Cl^- to particular cell types might not be a major factor contributing to salt tolerance. Further work with other genotypes that have similar shoot Cl^- concentrations, but differ in salt tolerance, could help determine if partitioning of Cl^- in epidermal cells, away from the mesophyll, contributes to salt tolerance. Another form of intercellular compartmentation of Cl^- in leaves is the accumulation of Cl^- in salt glands or bladders. These highly specialized cell structures are unique to some halophytes, which can accumulate Cl^- (and Na^+) in salt glands on leaf surfaces to lower internal leaf ion concentrations. Cl^- secretion via salt glands can be significant, with approximately 20% of leaf Cl^- excreted from salt glands of *L. fusca* at 100 mM NaCl [44]. A cation-chloride cotransporter (CCC) was localized to leaf trichomes and hydathodes in *Arabidopsis* [45] and was recently identified in salt-tolerant indica rice [46]. However, the exact role of these transporters in Cl^- transport is still not very clear. At the root level, Cl^- transport across different cell types from the cortex to the xylem could affect the total flux of Cl^- to the shoot [47].

Intracellular compartmentation of Cl^- is also important since low shoot concentrations of Cl^- and Na^+ are not always correlated with salt tolerance. Most species can "exclude" Cl^- and Na^+ up to 90–98%, [48], but salt tolerance is improved by efficient sequestration of Cl^- and Na^+ in vacuoles to prevent toxic level accumulation in the cytoplasm. Halophytes have mechanism for controlled uptake of Cl^- (and other ions) to maintain turgor-related growth and this relies on the effective sequestration of Cl^- into vacuoles [49, 50].

Plant cell vacuoles have capacity to accumulate up to 500 mM Cl^- [51]. In the halophyte *Suaeda maritima* growing at 200 mM NaCl, Cl^- concentrations ranged from 86 to 95 mM (cytoplasm), 430 to 465 mM (vacuole), and 111 to 130 mM (cell wall), with similar values found for Na^+ [52]. Using the fluorescent dye lucigenin, initial Cl^- transport into tonoplast vesicles from the salt-tolerant ice plant (*Mesembryanthemum crystallinum*) followed saturation kinetics, with a K_m of about 17 mM [53]. This K_m is significantly higher than those found for less salt-tolerant

species, for example, red beet (6.5 mM) [54]. The vacuolar Cl^- transport in the halophytic ice plant saturates at higher Cl^- concentrations than that for less salt-tolerant species [53]. In *Atriplex gmelini*, Cl^- concentrations of isolated vacuoles were 260 mM, which was almost the same as that measured in protoplasts, suggesting that for this halophyte, most of the leaf Cl^- (and Na^+) is compartmentalized into vacuoles [55]. For some species, there is indirect evidence that more efficient vacuolar sequestration of Cl^- is associated with salt tolerance as salt-tolerant genotypes of citrus, grapevine, and *Lotus* with low shoot Cl^- actually have higher root Cl^- concentrations compared to the more sensitive genotypes [47], suggesting more efficient compartmentation of Cl^- in root vacuoles for the tolerant genotypes. The efficient intracellular compartmentation of Cl^- is linked to salt tolerance. In a comparison between two maize genotypes that differ in salt tolerance, the more salt-sensitive genotype had consistently higher root cytoplasmic Cl^- concentrations than the tolerant genotype (based on ^{36}Cl flux analysis and electron microscopy) [52]. The estimates of cytoplasmic Cl^- concentrations were surprisingly high, with approximately 563 mM in the sensitive genotype and 360 mM for the tolerant genotype (measured at 100 mM NaCl) [52]. These values are consistent with other published estimates, as cytoplasmic Cl^- was also predicted to be about 350 mM for barley at 100 mM NaCl [38]. High ion concentrations in the cell wall may result in reduced turgor, leading to reduced shoot growth in the more sensitive genotype.

22.4 Amino Acid Transporters

One of the metabolic adaptations to high salinity in plants is the accumulation of compatible solutes [56]. Glycine betaine and proline are potent compatible solutes that are produced widely under salt/drought stress in flowering plants, such as Gramineae, Amaranthaceae, Malvaceae, and Compositae [57]. Within the amino acid transporter (ATF) superfamily, the amino acid permeases (AAPs) mediate proton-coupled uptake of structurally diverse amino acids, including proline. Proline transporters (ProTs) preferentially transport proline but no other amino acids [58, 59]. However, ProTs from tomato (LeProTs) were subsequently shown to transport both betaine and proline, although tomato is a betaine nonaccumulating plant [60, 61]. The homologous transporters were isolated from betaine accumulating mangrove *Avicennia marina* and were shown to transport betaine and proline [62]. So far, functional properties of ProTs have been reported in betaine nonaccumulating plants; *A. thaliana* (AtProT1–3) [58, 61], tomato (LeProT1–3) [60], and rice (OsProT1) [63], and betaine accumulating plants; and mangrove (AmBet/ProT1–2) [62], sugar beet (BvBet/ProT1) [64], and barley (HvProT1-2) [65, 66]. Among them, the selectivity of rice ProT for betaine remains uninvestigated [63], and the barley HvProT1 was reported to recognize proline, but not betaine [65]. All other transporters mediate transport of both betaine and proline.

Betainet is a small, nontoxic molecule that increases the osmotic potential of the cytoplasm without disturbing metabolism by stabilizing protein and membrane

structure against the damaging effect of high salinity [67]. Since betaine is a stable metabolic end product and not degraded in plants [68], accumulation patterns of betaine at cellular and plant levels are regulated by the synthesis and transport of betaine. In higher plants, betaine is synthesized by a two-step oxidation of choline [69]. The first step (choline, betaine aldehyde) is catalyzed by ferredox-independent choline monooxygenase in Amaranthaceae (e.g., spinach), while its corresponding enzyme in Gramineae (e.g., barley) has not been found [70, 71]. The last step is catalyzed by NAD^+-linked betaine aldehyde dehydrogenase (BADH) [72–74]. In Amaranthaceae, both steps are shown to be strictly localized in chloroplast stroma [75]. On the other hand, in barley, two *BADH* proteins are localized in peroxisomes and cytosol, respectively [76–78]. The accumulation of betaine is one of the adaptive strategies to adverse salt stress conditions [79, 80].

When betaine was applied exogenously to old leaves, it was translocated preferentially into young leaves and roots [64]. In response to salt stress, betaine levels increased in all tissues, but most significantly increased in young leaves. Expression of betaine transporter (BvBet/ProT1) was more strongly induced in old leaves than in young leaves, indicating importance of long-distance translocation of betaine [64].

γ-Aminobutyric acid (GABA) is a four-carbon nonprotein amino acid present in prokaryotes and eukaryotes. One *AtGAT1* (At1g08230) code for an H^+-driven, high-affinity GABA transporter from *Arabidopsis* was identified [81]. AtGAT1 did not transport betaine and proline. *AtGAT1* expression was highest in flowers and under conditions of elevated GABA concentrations such as wounding or senescence. GABA level increased under various stress conditions, such as low temperature, mechanical stimulation, and oxygen deficiency [82].

22.5 Sucrose Transporters

Sugars are essential as major energy source, substrates for polymer synthesis, storage compounds, and carbon precursors that are required for a large number of metabolic reactions in plants. Several transporters are required for distribution of sugars not only in the cells but also for long-distance transport [83]. Salinity stress enhances sugar accumulation in melon fruits [84]. In plants, soluble sugars are present in the form of glucose, fructose, and sucrose. Sucrose can be directly transported into sink cells or tissue or can be taken up by sucrose transporters or hexose transporters [85]. Under abiotic stress conditions, galactinol and raffinose accumulate in plant cells [86]. Overexpression of *GolS2*, an abiotic stress-inducible gene, increases galactinol and raffinose concentrations and improves abiotic stress tolerance in *Arabidopsis* [86]. It showed that sugars might also function as osmoprotectants; however, not much is known about their physiological function under abiotic stresses. The expression of genes is induced under abiotic stress conditions not only for sugar synthases but also for sugar transporters [87], and sugars might be transported to specific tissues or organelles under abiotic stress conditions [88]. Facilitated diffusion and secondary active transporters for sugars were found in plants [89–91]. They are mainly

secondary active transporters [88, 92–94], and in many cases the activities of secondary active transporters in plants depend on proton gradients. A plant sugar transporter from *Arabidopsis* STP1 was isolated and was observed to be a proton/hexose symporter localized at the plasma membrane. Two proton-dependent antiporters involved in glucose influx across the tonoplast were identified in *Arabidopsis* [88, 94]. From *Arabidopsis*, an abiotic stress-inducible transporter for monosaccharides from ERD6 family was isolated and named ESL1 [89]. Expression of *ESL1* was found to be induced under drought and high-salinity conditions and also with exogenous application of abscisic acid. *ESL1* is mainly expressed in pericycle and xylem parenchyma cells and was detected at tonoplast in transgenic *Arabidopsis* plants and tobacco BY-2 cells. The K_m for glucose uptake activity of mutated ESL1 in the transgenic BY-2 cells was extraordinarily high, and the transport activity was independent of a proton gradient, indicating that ESL1 is a low-affinity, facilitated diffusion transporter. It was reported that ESL1 might function coordinately with the vacuolar invertase to regulate osmotic pressure by affecting the accumulation of sugar in plant cells [89].

22.6 Transporters in Rice and *Arabidopsis*

When a comparison is made between numbers of membrane transporter genes in *Arabidopsis* and rice, many transporter genes are found to be similar in these plants; *Arabidopsis* has a more diverse array of genes for multiefflux transport and for response to stress signals, and rice has more secondary transporter genes for carbohydrate and nutrient transport [2]. In rice, a total of 1200 membrane transporters are identified, among which 800 are secondary transporters, 180 are pumps, and 120 are channel transporters. In pumps, 153 are ABC and 57 are P-ATPase kinds of transporters; in channels, MIPs are maximum 38, Hsp are 23, 14 CIC, and annexins 10; and in secondary transporters, MFS are 145, MC 56, DMT 57, CpA2 16, and CACA 23 [2]. Whole-genome expression profiling was examined to find out transporters under salt and drought stress conditions in indica rice.

Microarrays were used to identify differentially expressed genes of rice in response to salt stress for shoots [95] and roots [96] and to compare rice with other cereals [97]. Problem with these approaches was that they also identified many false positives because of treatment and the use of different species. To minimize these risks, the use of cultivars with different salt sensitivity for comparative transcriptomics studies was used for salt and drought stress [98]. For salinity, CSR-11 (salt tolerant) and VSR-156 (salt sensitive), as well as for drought Vandana (tolerant) and IR-64 (sensitive), were used to identify transporters involved in both stresses. Expression profiling was done and presented as heat map (Figure 22.4) with a list of 72 genes that included all the transporters expressed differentially. *Arabidopsis* microarray data for salinity stress and drought stress were used. All differentially expressed transporters were extracted after analysis ($p < 0.05$) and with a fold change >2. False discovery rates were kept at <2% and heat map for 127 genes for transporters was presented (Figure 22.5). Venn

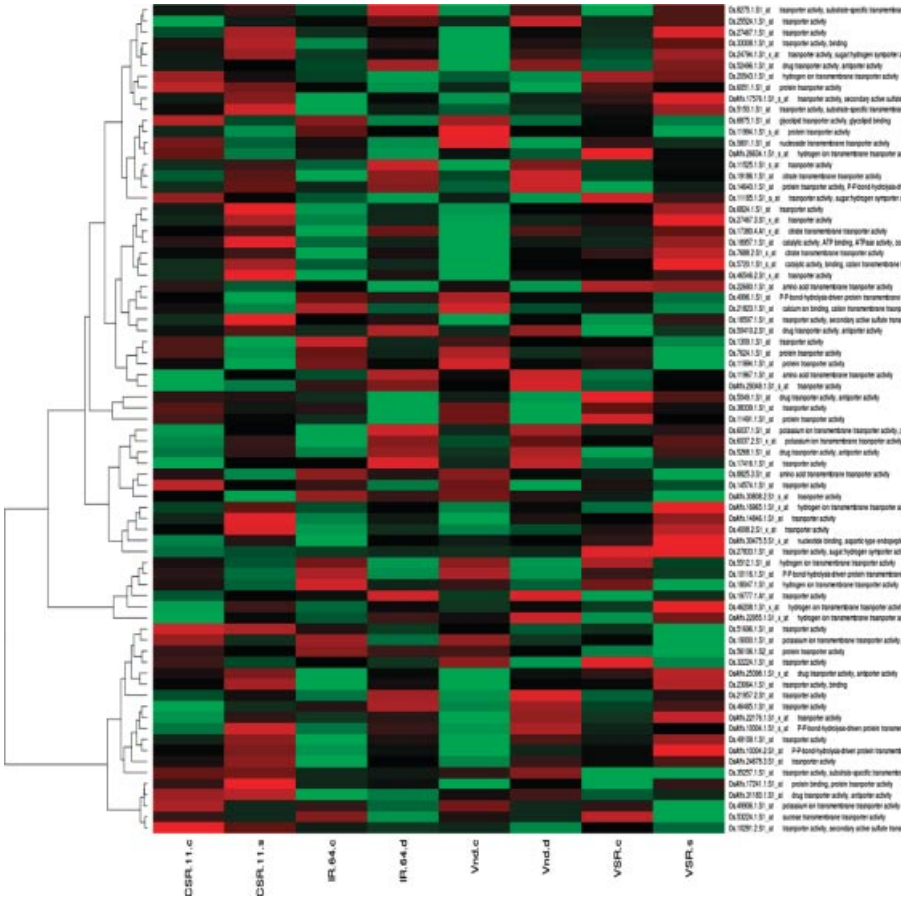

Figure 22.5 The expression profiling of transporters differentially expressed under salt-tolerant (CSR-11), salt-sensitive (VSR), drought-tolerant (Vandana), and drought-sensitive (IR-64) conditions using hierarchical clustering of 70 genes.

diagram (Figure 22.6) for comparing the number of expressed genes under salt and drought conditions showed that 52 genes were common to both stresses that include mainly metal ion, peptide, amino acids Na^+/H^+, sugar, cadmium/zinc, peptide, cation transporter HKT1, phosphatidylinositol transfer-like protein III, aquaporin TIP2.2, and high-affinity cationic amino acid transporters. QTL analyses using rice (*Oryza sativa*) plants identified a gene contributing to salt tolerance of an indica rice cultivar Nona Bokra. The gene encoded a Na^+ transporter, named SKC1 or OsHKT1;5 [28], which is an orthologue of AtHKT1;1. OsHKT1;5 was found to function in Na^+ exclusion from xylem [28]. Two major QTL controlling Na^+ exclusion from leaves in wheat were also identified, which conferred salt tolerance encoding the AtHKT1;1 orthologues HKT1;4 and HKT1;5 [99, 100]. The common requirement of a HKT-mediated Na^+ exclusion from the xylem in *Arabidopsis*, rice, and wheat

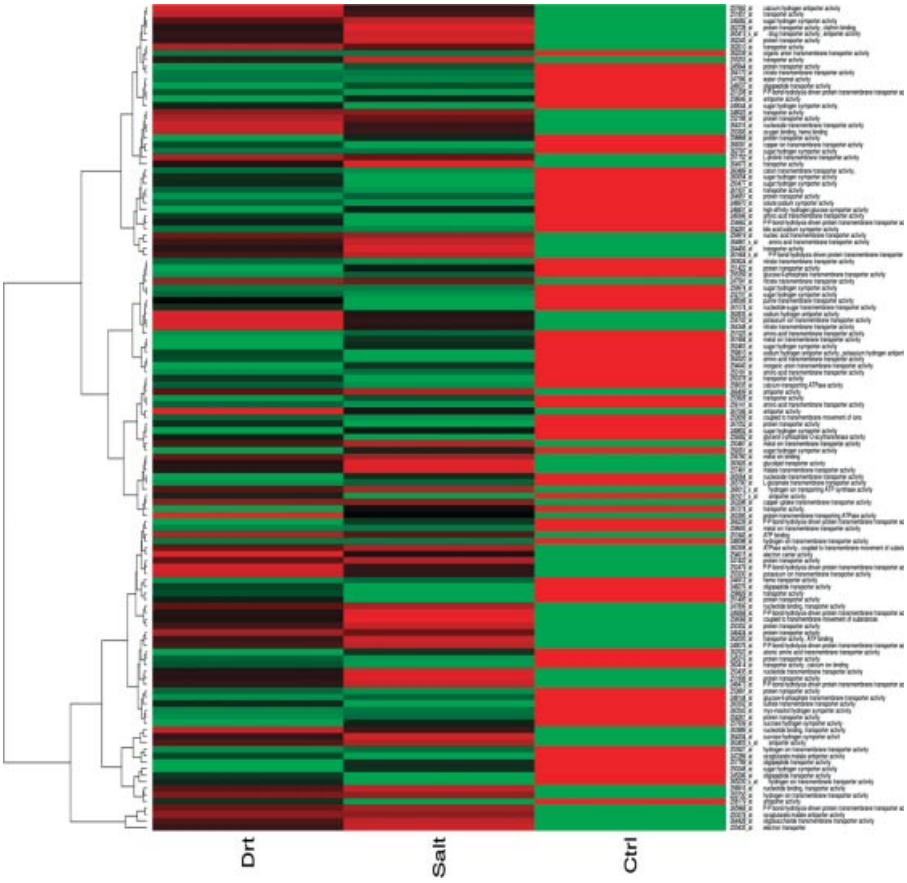

Figure 22.6 The expression profiling of transporters differentially expressed under salt and drought conditions in *Arabidopsis* using hierarchical clustering of 127 genes.

recommended that this is a widely conserved primary salt tolerance mechanism in glycophytes [101]. Rice, a monocot model, contains nine HKT-like genes, which is different from dicot model plant *Arabidopsis* that has a single AtHKT1 [102]. In the case of *Arabidopsis*, 40 genes were common to salt and drought stress conditions (Figure 22.7), which were mainly water channels, ABC transporters, amino acid transmembrane transporter, sugar:hydrogen ion symporter, oligopeptide transporter, antiporter/glucose-6-phosphate transmembrane transporter, oligopeptide transporter, oxoglutarate:malate antiporter, copper ion transmembrane transporter, coupled to transmembrane movement of ions, and sodium:hydrogen antiporter.

The rice genome size (430 Mb) is more than three times that of *Arabidopsis* (125 Mb), but the total number of membrane transporter proteins (1200) is only 1.20 times that in *Arabidopsis* (1000). The total number of differentially expressed transporters under salt and drought stress is 135 in rice, while it was 145 in *Arabidopsis*. Being a wild plant, *Arabidopsis* lives under diverse soil conditions and under environmental

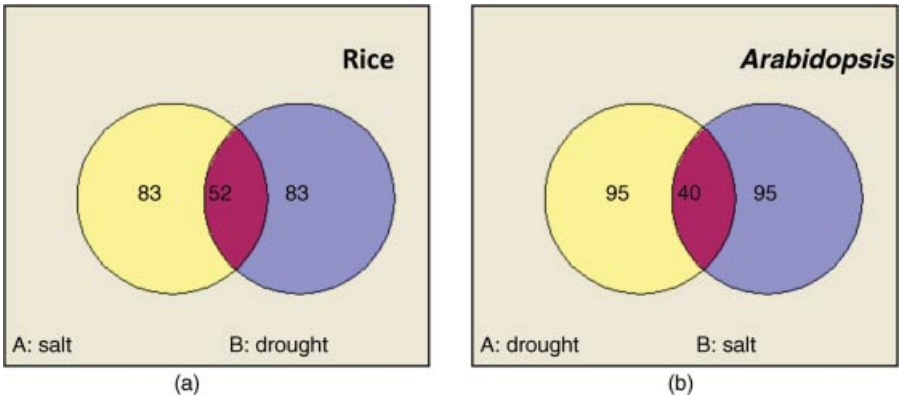

Figure 22.7 (a) Venn diagram comparing transporters of rice expressed under salt and drought stress. (b) Venn diagram comparing transporters of *Arabidopsis* expressed under salt and drought stress.

stress, whereas rice is a cultivated crop plant grown under more stable environmental conditions. Therefore, *Arabidopsis* has diverged more than the rice to form transporter systems involved in multiefflux and stress response signaling. However, rice has more pump and secondary transporter genes (ABC, P-type ATPase, MFS, and POT) for carbohydrate and nutrient transport systems [2]. Amino acid (AAAP), ammonia (Amt), sulfate (SulP), metal ion (ZIP), Ca^{2+} (CaCA), and K^+ transport protein gene families (VIC, KUP) are specifically diverged in rice due to nutrient acquisition growth in subtropical plants. It was found that HKT transporters, HKT6, 8, 11, 12, 14, 16, and 17, are differentially expressed. The genome of the japonica rice cultivar Nipponbare has been reported to contain nine *OsHKT* genes with two of them being nonfunctional [102]. Among the remaining seven OsHKT transporters, OsHKT1;5 was found to be an AtHKT1;1 orthologue [102]. A phylogenetic analysis using the amino acid sequences of HKT transporters has shown that the HKT transporters are of two major subfamilies, class I and class II [103]. Remarkable differences between these two classes of HKT transporters were found in the structure of the putative selectivity pore-forming regions and the selectivity for K^+ characterized in heterologous expression systems. HKT transporters are members of a large K^+ transporter family, the HKT/Trk/Ktr family, which retains four selectivity pore-forming regions (p-loops) that show similarity to a bacterial K^+ channel [104, 105]. It was found that a serine to glycine replacement in the first p-loop region of AtHKT1;1 and OsHKT2;1 transporters that show a poor K^+ permeability conferred a robust K^+ permeability, while a glycine to serine replacement abolished the robust K^+ permeability from typical class II transporters TaHKT2;1 and OsHKT2;2 [106]. The OsHKT2;1 transporter is an unusual class II HKT transporter. It is clearly classified into the HKT2 transporter subfamily in sequence analyses [103]. The OsHKT2;1 is a SGGG type, but not a class II representative GGGG-type transporter, and shows a strong preference for Na^+-selective transport in yeast and *X. laevis* oocytes, comparable to typical class I HKT transporters [2, 11].

22.7 Conclusions

The outline above indicates the importance of plant transporters for abiotic stresses. Genomics, proteomics, and molecular functional approaches, coupled with a strong bioinformatics capability, now can be used efficiently for the study of plant transporters involved in abiotic stresses. This understanding of plant transporters can be used for the manipulation of the responses or their transfer to important cereal crop species through conventional, marker-assisted, or transgenic approaches.

References

1 FAO (2005) Food and Agriculture Organization of United Nations.
2 Nagata, T., Iizumi, S., Satoh, K., *et al.* (2008) *Plant Mol. Biol.*, **66**, 565–585.
3 Munns, R. and Tester, M. (2008) *Annu. Rev. Plant Biol.*, **59**, 651–681.
4 Rajendran, K., Tester and, M., and Roy, S.J. (2009) *Plant Cell Environ.*, **32**, 237–249.
5 Wu, S.J., Ding, L., and Zhu, J.K. (1996) *Plant Cell*, **8**, 617–627.
6 Apse, M., Aharon, G., Sneddon, W., *et al.* (1999) *Science*, **285**, 1256–1258.
7 Gaxiola, R.A., Li, J., Undurraga, S., *et al.* (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 11444–11449.
8 Shi, H., Ishitani, M., Kim, C., *et al.* (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 6896–6900.
9 Uozumi, N., Kim, E.J., Rubio, F., *et al.* (2000) *Plant Physiol.*, **121**, 1249–1259.
10 Mäser, P., Eckelman, B., Vaidyanathan, R., *et al.* (2002) *FEBS Lett.*, **531**, 157–161.
11 Hori, T. and Schroeder, J.I. (2004) *Plant Physiol.*, **136**, 2457–2462.
12 Apse, M.P. and Blumwald, E. (2007) *FEBS Lett.*, **581**, 2247–2254.
13 Shi, H., Quintero, F.J., *et al.* (2002) *Plant Cell*, **4**, 465–477.
14 Shabala, L., Cuin, T.A., Newman, I.A., *et al.* (2005) *Planta*, **222**, 1041–1050.
15 Schachtman, D.P. and Schroeder, J.I. (1994) *Nature*, **370**, 655–658.
16 Berthomieu, P., Conejero, G., Nublat, A., *et al.* (2003) *EMBO J.*, **22**, 2004–2014.
17 Gong, H.J., Randall, D.P., and Flowers, T.J. (2006) *Plant Cell Environ.*, **29**, 1970–1979.
18 Sunarpi, H.T., Motoda, J., Kubo, M., *et al.* (2005) *Plant J.*, **44**, 928–938.
19 Horie, T., Horie, R., Chan, W.Y., *et al.* (2006) *Plant Cell Physiol.*, **47**, 622–633.
20 Lin, C.C. and Kao, C.H. (2001) *Plant Soil*, **237**, 165–171.
21 Kinraide, T.B. (1999) *J. Exp. Bot.*, **50**, 1495–1505.
22 Husain, S., von Caemmerer, S., and Munns, R. (2004) *Funct. Plant Biol.*, **31**, 1115–1126.
23 Plett, D.C. and Møller, I.S. (2010) *Plant Cell Environ.*, **33**, 612–626.
24 Luo, Q., Bingjun, Y., and Liu, Y. (2005) *J. Plant Physiol.*, **162**, 1003–1012.
25 Cramer, G.R. (2002) Sodium-calcium interactions under salinity stress, in *Salinity. Environment-Plants-Molecules* (eds A. Läuchli and U. Lüttge), Kluwer, Dordrecht, pp. 205–227.
26 Demidchik, V., Essah, P.A., and Tester, M. (2004) *Planta*, **219**, 167–175.
27 Kaplan, B., Sherman, T., and Fromm, H. (2007) *FEBS Lett.*, **581**, 2237–2246.
28 Ren, Z.-H., Gao, J.-P., Li, L.-G., *et al.* (2005) *Nat. Genet.*, **37**, 1141–1146.
29 Amtmann, A., Troufflard, S., and Armengaud, P. (2008) *Physiol. Plant.*, **133**, 682–691.
30 Spalding, E.P., Hirsch, R.E., Lewis, D.R., *et al.* (1999) *J. Gen. Physiol.*, **113**, 909–918.
31 Duby, G., Hosy., E., Fizames., C., Alcon, C., *et al.* (2008) *Plant J.*, **53**, 115–123.

32 Jeanguenin, L., Lebaudy, A., Xicluna, J., *et al.* (2008) *Plant Signal. Behav.*, **3**, 622–625.

33 Gaymard, F., Pilot, G., Lacombe, B., Bouchez, D., *et al.* (1998) *Cell*, **94**, 647–655.

34 Lebaudy, A., Vavasseur, A., Hosy, E., *et al.* (2008) *Proc. Natl. Acad. Sci. USA*, **105**, 5271–5276.

35 Teakle, N. and Tyerman, S.D. (2010) *Plant Cell Environ.*, **33**, 566–589.

36 Bhatti, A.S. and Wieneke, J. (1984) *J. Plant Nutr.*, **7**, 1233–1250.

37 Yamashita, K. and Matsumoto, H. (1996) *Soil Sci. Plant Nutr.*, **42**, 209–213.

38 Britto, D.T., Ruth, T.J., Lapi, S., *et al.* (2004) *Planta*, **218**, 615–622.

39 Boursier, P. and Lauchli, A. (1989) *Physiol. Plant.*, **77**, 537–544.

40 Lorenzen, I., Aberle, T., and Plieth, C. (2004) *Plant J.*, **38**, 539–544.

41 Sun, J., Chen, S.L., Dai, S.X., *et al.* (2009) *Plant Physiol.*, **149**, 1141–1153.

42 Huang, C.X. and Van Steveninck, M.E. (1989) *Plant Physiol.*, **90**, 1440–1443.

43 James, R., Davenport, R., and Munns, R. (2006) *Plant Physiol.*, **142**, 537–1547.

44 Jeschke, W., Klagges, S., Hilpert, A., *et al.* (1995) *New Phytol.*, **130**, 23–35.

45 Colmenero-Flores, J.M., Martinez, G., Gamba, G., *et al.* (2007) *Plant J.*, **50**, 278–292.

46 Pandit, A., Rai, V., Bal, S., *et al.* (2010) *Mol. Genet. Genomics*, **284**, 121–136.

47 Storey, R., Schachtman, D.P., and Thomas, M.R. (2003) *Plant Cell Environ.*, **26**, 789–800.

48 Munns, R. (2005) *New Phytol.*, **167**, 645–663.

49 Flowers, T.J., Troke, P.F., and Yeo, A.R. (1977) *Annu. Rev. Plant Physiol.*, **28**, 89–121.

50 Glenn, E.P., Brown, J.J., and Blumwald, E. (1999) *Crit. Rev. Plant Sci.*, **18**, 227–255.

51 Cram, W.J. (1973) *J. Exp. Bot.*, **24**, 328–341.

52 Hajibagheri, M.A., Yeo, A.R., Flowers, T.J., *et al.* (1989) *Plant Cell Environ.*, **12**, 753–757.

53 Wissing, F. and Smith, J.A.C. (2000) *J. Membr. Biol.*, **177**, 199–208.

54 Pople, A.J. and Leigh, R.A. (1988) *Planta*, **181**, 406–413.

55 Matoh, T., Watanabe, J., and Takahashi, E. (1987) *Plant Physiol.*, **84**, 173–177.

56 Rhodes, D. and Hanson, A.D. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **44**, 357–384.

57 McNeil, S.D., Nuccio, M.L., and Hanson, A.D. (1999) *Plant Physiol.*, **120**, 945–949.

58 Rentsch, M., Hirner, B., Schmelzer, E., *et al.* (1996) *Plant Cell*, **8**, 1437–1446.

59 Rentsch, D., Boorer, K.J., and Frommer, W.B. (1998) *J. Membr. Biol.*, **162**, 177–190.

60 Schwacke, R., Grallath, S., Breitkreuz, K.E., *et al.* (1999) *Plant Cell*, **11**, 377–391.

61 Grallath, S., Weimar, T., Meyer, A., *et al.* (2005) *Plant Physiol.*, **137**, 117–126.

62 Waditee, R., Hibino, T., Tanaka, Y., *et al.* (2002) *J. Biol. Chem.*, **277**, 18373–18382.

63 Igarashi, Y., Yoshiba, Y., Takeshita, T., *et al.* (2000) *Plant Cell Physiol.*, **41**, 750–756.

64 Yamada, N., Promden, W., Yamane, K., *et al.* (2009) *J. Plant Physiol.*, **166**, 2058–2070.

65 Ueda, A., Shi, W., Sanmiya, K., *et al.* (2001) *Plant Cell Physiol.*, **42**, 1282–1289.

66 Fujiwara, T., Mitsuya, S., Miyake, H., *et al.* (2010) *Planta*, **232**, 133–143.

67 Nomura, M., Hibino, T., Takabe, T., *et al.* (1998) *Plant Cell Physiol.*, **39**, 425–432.

68 Ladyman, J.A.R., Hitz, W.D., and Hanson, A.D. (1980) *Planta*, **150**, 191–196.

69 Hanson, A.D., Rathinasabapathi, B., and Rivoal, J. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 306–310.

70 Brouquisse, R., Weigel, P., Rhodes, D., *et al.* (1989) *Plant Physiol.*, **90**, 322–329.

71 Hibino, T., Waditee, R., Araki, E., *et al.* (2002) *J. Biol. Chem.*, **277**, 41352–41360.

72 Weigel, P., Weretilnyk, E.A., and Hanson, A.D. (1986) *Plant Physiol.*, **82**, 753–759.

73 Arakawa, K., Katayama, M., and Takabe, T. (1990) *Plant Cell Physiol.*, **31**, 797–803.

74 Hibino, T., Meng, Y.L., Kawamitsu, Y., *et al.* (2001) *Plant Mol. Biol.*, **45**, 353–363.
75 Hanson, A.D., May, A.M., Grumet, R., Bode, J., *et al.* (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3678–3682.
76 Ishitani, M., Nakamura, T., Han, S.Y., *et al.* (1995) *Plant Mol. Biol.*, **27**, 307–315.
77 Nakamura, T., Nomura, M., Mori, H., *et al.* (2001) *Plant Cell Physiol.*, **42**, 1088–1092.
78 Nakamura, T., Yokota, S., Muramoto, Y., *et al.* (1997) *Plant J.*, **11**, 1115–1120.
79 Waditee, R., Bhuiyan, M.N., Rai, V., *et al.* (2005) *Proc. Natl. Acad. Sci. USA*, **102**, 1318–1323.
80 Waditee, R., Bhuiyan, N.H., Hirata, E., *et al.* (2007) *J. Biol. Chem.*, **282**, 34185–34193.
81 Meyer, A., Eskandari, S., Grallath, S., *et al.* (2006) *J. Biol. Chem.*, **281**, 7197–7204.
82 Bouché, N. and Fromm, H. (2004) *Trends Plant Sci.*, **9**, 110–115.
83 Lalonde, S., Wipf, D., and Frommer, W.B. (2004) *Annu. Rev. Plant Biol.*, **55**, 341–372.
84 Ozaki, K., Uchida, A., Takabe, T., *et al.* (2009) *J. Plant Physiol.*, **166**, 569–578.
85 Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006) *Annu. Rev. Plant Biol.*, **57**, 675–709.
86 Taji, T., Ohsumi, C., Iuchi, S., *et al.* (2002) *Plant J.*, **29**, 417–426.
87 Maruyama, K., Sakuma, Y., Kasuga, M., *et al.* (2004) *Plant J.*, **38**, 982–993.
88 Wormit, A., Trentmann, O., Feifer, I., *et al.* (2006) *Plant Cell*, **18**, 3476–3490.
89 Yamada, K., Osakabe, Y., Mizoi, J., *et al.* (2010) *J. Biol. Chem.*, **285**, 1138–1146.
90 Martinoia, E., Kaiser, G., Schramm, M.J., *et al.* (1987) *J. Plant Physiol.*, **131**, 467–478.
91 Sacchi, G., Abruzzese, A., Lucchini, A., *et al.* (2000) *Plant Soil*, **220**, 1–11.
92 Sauer, N., Friedländer, K., and Gräml-Wicke, U. (1990) *EMBO J.*, **9**, 3045–3050.
93 Klepek, Y.S., Geiger, D., Stadler, R., *et al.* (2005) *Plant Cell*, **17**, 204–218.
94 Aluri, S. and Buttner, M. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 2537–2542.
95 Zhou, J., Wang, X., Jiao, Y., *et al.* (2007) *Plant Mol. Biol.*, **63**, 591–608.
96 Kawasaki, S., Borchert, C., Deyholos, M., *et al.* (2001) *Plant Cell*, **13**, 889–905.
97 Ueda, A., Kathiresan, A., Bennett, J., *et al.* (2006) *Theor. Appl. Genet.*, **112**, 1286–1294.
98 Senadheera, P., Singh, R.K., and Maathuis, F.J.M. (2009) *J. Exp. Bot.*, **60**, 2553–2563.
99 Huang, S., Spielmeyer, W., Lagudah, E.S., *et al.* (2006) *Plant Physiol.*, **142**, 1718–1727.
100 Byrt, C.S., Platten, J.D., Spielmeyer, W., *et al.* (2007) *Plant Physiol.*, **143**, 1918–1928.
101 Horie, T., Hauser, F., and Schroeder, J.I. (2009) *Trends Plant Sci.*, **14**, 660–668.
102 Garciadeblás, B., Senn, M., Banuelos, M., *et al.* (2003) *Plant J.*, **34**, 788–801.
103 Platten, J.D., Cotsaftis, O., Berthomieu, P., *et al.* (2006) *Trends Plant Sci.*, **11**, 372–374.
104 Durell, S.R. and Guy, H.R. (1999) *Biophys. J.*, **77**, 789–807.
105 Kato, N., Akai, M., Zulkifli, L., *et al.* (2007) *Channels*, **1**, 161–171.
106 Mäser, P., Hosoo, Y., Goshima, S., *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 6428–6433.

23
Potassium and Sodium Transporters: Improving Salinity Tolerance in Plants

Toshio Yamaguchi, Nobuyuki Uozumi, and Tomoaki Horie

Crop productivity can be greatly affected by soil salinity since high concentrations of Na^+ cause osmotic stress and ion-specific cytotoxicity in most plant species. Plants have developed multiple mechanisms to alleviate Na^+ stresses, which include exporting Na^+ from the cells, compartmentation to vacuoles, and preventing Na^+ from being transported to photosynthetic organs such as leaves. Recent advancements in molecular and genetic studies have led to the identification of several classes of ion transporters that are involved in these processes and have extended our understanding of the molecular mechanisms that plants use to circumvent Na^+ toxicity. Among these, the NHX-type tonoplast cation/H^+ antiporters, the HKT-type plasma membrane Na^+(K^+) transporters, and the SOS1-type plasma membrane Na^+/H^+ antiporters have drawn particular attention due to their physiological significance under salinity stress. Moreover, ectopic tissue-specific overexpression of these molecules has provided promising evidence for the utility of a transgenic approach toward improving crop salinity tolerance using genes encoding Na^+ transporters.

23.1
Introduction

Soil salinity imposes severe deleterious effects on crop productivity in most plant species, mainly through osmotic stress and ion (Na^+)-specific toxicity [1–3]. The accumulation of sodium cations (Na^+) in the cytosol causes serious damage via inhibition of essential cellular processes, including protein synthesis [4] and vital enzyme reactions [5, 6]. Moreover, since photosynthetic processes have been shown to be susceptible to salinity [7], it is important that photosynthetic organs are protected from overaccumulation of Na^+ in order to maintain efficient carbon

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fixation and biomass production [8]. Na^+ is not an essential mineral ion in plants, and removal of excessive Na^+ from the cells is necessary to reduce Na^+ toxicity. Recent studies have revealed that some classes of Na^+ transporters play crucial roles in Na^+ homeostasis during salinity stress [9–11]. Among them, the *Arabidopsis* plasma membrane Na^+/H^+ antiporter SOS1 (salt overly sensitive 1), the Na^+ transporter AtHKT1;1, and the tonoplast Na^+/H^+ antiporter AtNHX1 have drawn particular interest due to their capacity to transport Na^+, and their molecular functions and physiological roles have been extensively studied. These Na^+ transporters have been described in a number of plant species, and they have been found to contribute to Na^+ extrusion, limited Na^+ accumulation in leaves, and monovalent cation sequestration in plant vacuoles to alleviate sodium stress under saline conditions. In addition, some of these transporters have been shown to improve salinity tolerance of crop plants when their encoding gene is overexpressed.

In this chapter, we will provide an overview of the molecular properties, regulation mechanisms, and physiological roles of distinct classes of transporters that are involved in plant Na^+ tolerance, with particular emphasis on NHX-, HKT-, and SOS1-type transporters. We will also summarize the advances in transgenic approaches using these molecules and discuss the potential for using other K^+ or Na^+ transporters in the improvement of plant salinity tolerance.

23.2 NHX Transporters

23.2.1 Overview

One of the mechanisms that plant cells use to cope with Na^+ stress is compartmentation of Na^+ into the vacuoles, and many reports have indicated that Na^+/H^+-exchanging NHX transporters are involved in this process (Figure 23.1) [2]. The plant Na^+/H^+ exchanger NHX was first identified in *Arabidopsis* via a complementation study using the *Δnhx1* yeast mutant [12], and NHX-type transporters have since been identified in a wide variety of plant species [13–16]. In general, plant genomes contain genes encoding more than one type of NHX isoform; for example, six NHX isoforms have been identified in *Arabidopsis*, rice, and maize (AtNHX1–6, OsNHX1–6, and ZmNHX1–6, respectively), four isoforms in tomato (LeNHX1–4), and at least two isoforms in Japanese morning glory (*Ipomoea nil*; InNHX1-2). Most of the NHX proteins studied to date have been shown to localize to the endomembranes including the tonoplast (see below) and mediate Na^+/H^+ and K^+/H^+ antiport across the membrane by utilizing the H^+ gradient as a driving force, as demonstrated using a yeast heterologous expression system [12, 17–20], isolated plant vacuoles [21], and proteoliposomes containing purified NHX proteins [22, 23].

Phylogenetic analysis has revealed that NHX family transporters belong to the cation/proton antiporter 1 (CPA1) family according to the transporter classification (TC) system [24]. The NHX family includes a distinct subfamily called the IC-NHE/NHX

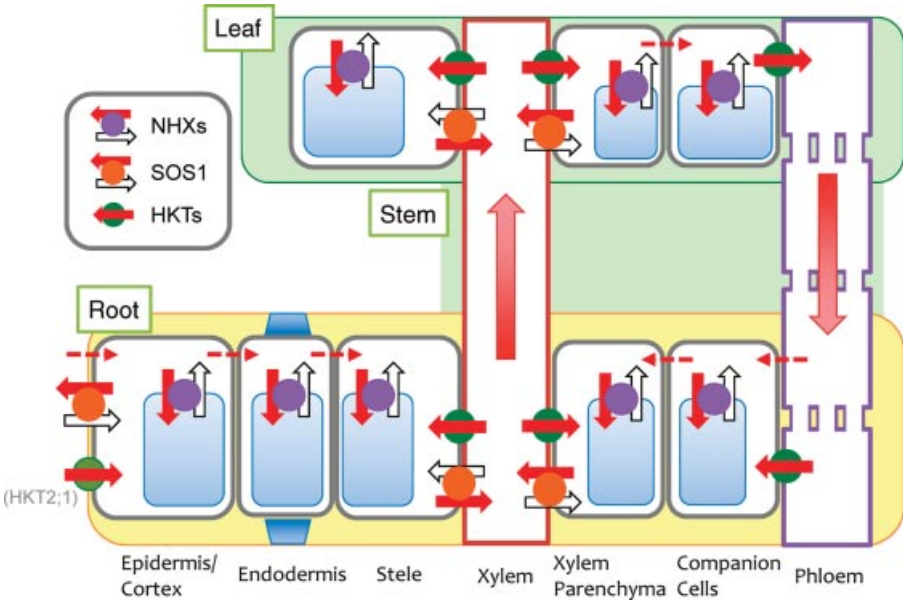

Figure 23.1 Na^+ movement in plant tissues and the involvement of NHX-, SOS1-, and HKT-type transporters in Na^+ detoxification under salinity stress. Na^+ entering through the plasma membrane of the root epidermis is exported out of the cells by SOS1-type transporters or compartmentalized into the vacuole via NHX-type transporters in order to maintain a low cytosolic Na^+ concentration [18, 19, 21, 25, 111, 112, 115, 117]. Excess Na^+ that reaches stele through radial movement is loaded into the xylem via SOS1-type transporters [116]. HKT-type transporters in xylem parenchyma cells function in removing Na^+ from the xylem stream to protect shoots from overaccumulating Na^+ and also in loading Na^+ into the phloem for transporting it back to the roots (Na^+ recirculation) [64, 65, 67, 70, 85–88, 90, 104]. A low cytosolic Na^+ concentration in leaves is achieved through the actions of SOS1- and NHX-type transporters. Note that HKT2;1 transporters have been shown to be a Na^+ influx transporter in wheat [98] and rice [102] when extracellular K^+ availability is limited.

family composed of intracellular (IC) cation/H^+ antiporters [14]. The members of IC-NHE/NHX subfamily can be further subdivided into two groups, namely, class I and class II [14, 15], although the functional relevance of this subdivision is yet to be studied. The tonoplast localization of class I NHXs in various plant species has been well documented using subcellular fractionation [17, 25–28], transient or stable expression of GFP fusion proteins [17, 19, 29, 30], and immunomicroscopic analysis [28]. On the other hand, subcellular localization of a class II NHX was studied by expressing a GFP fusion protein of LeNHX2 in onion epidermal cells, and it indicated a nonvacuolar vesicular localization reminiscent of yeast ScNHX1 [12, 31, 32].

The spatial localization pattern of *NHX* transcripts has been studied in several plant species, indicating diverse expression patterns of NHX isoforms. In rice, *OsNHX1* expression was found in whole tissues, although the expression was higher in shoots than in roots [17]. *MNhx* transcripts of the ice plant *Mesembryanthemum*

crystallinum were found in leaves, stems, flowers, seedpods, weakly in roots [33]. In *Arabidopsis*, *AtNHX1* and *AtNHX2* were the most abundant isoforms and transcripts were widespread across roots and shoots, whereas expression of *AtNHX3* and *AtNHX4* was almost exclusively detected in flowers and roots, albeit in lower amounts than that of *AtNHX1* and *AtNHX2* [18, 19]. *AtNHX5* transcripts were found in all organs tested, although the expression level was lower than that of *AtNHX1* or *AtNHX2* [18, 19]. The expression of *AtNHX6* was found in roots and shoots, but the amount of transcripts was very low [19]. *InNHX1* and *InNHX2* genes of *I. nil* were expressed in virtually all organs tested. *InNHX1* expression is extensively induced in floral organs (particularly in flower limbs at 12 h before flowering), with induction being necessary for flower color change during maturation [20, 29]. It might be too early to generalize, but these results imply the possibility that some NHX isoforms are ubiquitously expressed and play housekeeping roles, while other isoforms have specific expression patterns for more specialized roles in the tissues where they are expressed.

Hydropathy profiling of NHX proteins suggests the presence of 10–12 transmembrane segments and a relatively long C-terminal hydrophilic tail [34]. At present, the structural properties of NHXs have been studied only for AtNHX1, and two distinct topology models have been proposed. The first topology model was proposed based on the results of protease protection assays of yeast vacuoles expressing AtNHX1 proteins with triple hemagglutinin ($3 \times$ HA) epitope tags inserted into the hydrophilic loops [34]. The proposed model was unlike any model for known Na^+/H^+ antiporters and consisted of nine transmembrane segments with three membrane-associated hydrophobic regions that would not span a membrane (TM3, 5, and 6) and a C-terminal hydrophilic tail facing the vacuolar lumen [34]. The C-terminus hydrophilic region of Na^+/H^+ antiporters had been shown to be involved in the regulation of transport activity in mammalian NHE1 [35, 36] and ApNhaP from the cyanobacterium *Aphanothece halophytica* [37]. In both cases, the C-terminal tails were exposed to the cytoplasm and were therefore considered to be involved in sensing of the cytoplasmic pH and cation environment. The regulation of transport activity via the C-terminus has also been suggested for AtNHX1; truncation of the C-terminus region of AtNHX1 resulted in increased Na^+ transport without affecting K^+ transport activity in yeast, suggesting that the C-terminal tail plays a role in modulating cation selectivity of AtNHX1 [34]. However, it should be noted that regulation of transport activity according to this model is supposed to occur from the inside of the vacuole rather than from the cytosolic side, as in the case of the NHE1 and ApNhaP antiporters. In addition, it has been shown that another regulatory protein is involved in the regulation of AtNHX1 activity via the C-terminus. Yeast two-hybrid screening identified a calmodulin-like protein, AtCaM15, as a protein interacting with the AtNHX1 C-terminus. This was confirmed by a pull-down assay using a yeast expression system and affinity purified recombinant proteins [38]. Coexpression of AtCaM15 with AtNHX1 decreased Na^+ transport activity in yeast vacuoles, which suggested the involvement of AtCaM15 in the modulation of AtNHX1 cation selectivity. AtCaM15 was found inside the vacuoles of yeast and plant cells overexpressing AtCaM15, which further supports the above topology

model. Interestingly, it was shown that the interaction between AtCaM15 and AtNHX1 was enhanced as the pH became more acidic. These results indicate that, at physiological conditions, AtCaM15 would be bound to the AtNHX1 C-terminus and AtNHX1 would favor K^+/H^+ antiport over Na^+/H^+ antiport due to the acidic vacuolar luminal pH [38]. In addition, there are several reports that have shown increased vacuolar pH under salinity stress [39, 40]. Such a pH change would cause disassociation of AtCaM15 and AtNHX1, leading to increased Na^+/H^+ antiport and compartmentation of Na^+ into the vacuoles under salinity stress.

Another topology model has been proposed on the basis of *in vitro* translation experiments using truncated *AtNHX1* genes. Sato and Sakaguchi [41] showed that several transmembrane segments of AtNHX1 retain topogenic properties similar to human NHE1 and suggested that AtNHX1 has a topology similar to human NHE1, consisting of 11 transmembrane segments and an intramembranous hydrophobic region (TM9) with the C-terminal tail exposed to the cytoplasm [41, 42].

23.2.2 Physiological Roles of NHXs

It has been well established that NHX isoforms mediate Na^+/H^+ and K^+/H^+ antiport and compartmentation of Na^+ and K^+ into the vacuole (or intracellular vesicles) at a cellular level (Figure 23.1); however, there are few experimental data to clarify the physiological roles of NHX isoforms. The physiological roles of *NHX* isoforms can be deduced from their spatial expression patterns (described above) and, more important, from the stress-specific induction of each *NHX* isoform. Although most of the *NHX* isoforms have been shown to be expressed under nonstress conditions to some extent, the stress-specific expression of several isoforms has also been reported. *AtNHX1*, *AtNHX2*, and *AtNHX5* were induced by salt and osmotic stresses [19, 43], indicating the potential roles of NHX isoforms under these stresses. Rice *OsNHX1* expression is also induced by salt and osmotic stress, although the induction by osmotic stress was less than that by salt, indicating that ionic stress is mainly responsible for this induction [17]. The induction of *NHX* transcripts by salt stress has also been observed for *MNhx* in the ice plant and *InNHX2* in Japanese morning glory [29, 33]. These results suggest a general role for NHXs under salt stress and, in some cases, under osmotic stress.

In addition to its role in stress response, the involvement of an NHX isoform, InNHX1, in Japanese morning glory in the regulation of vacuolar pH has been well documented; a natural variant of *I. nil* that has a transposon insertion in the *InNHX1* locus shows a marked reduction in vacuolar pH of flower petal cells compared to wild type, resulting in purple flower coloration in the mutant as opposed to the blue wild-type flowers. Furthermore, specific induction of *InNHX1* during flower opening has been observed in wild-type petals [20]. Developmental stage-specific induction has also been observed in grape (*Vitis vinifera*) *VvNHX1* during fruit maturation [44]. These results indicate the involvement of NHX isoforms in diverse physiological roles, including cellular ion homeostasis, stress tolerance, and even organ development.

Characterization of a T-DNA insertion mutant of AtNHX1 (*atnhx1*) revealed that this line is indeed susceptible to salinity, as the detrimental effects of sublethal concentrations of NaCl on seedling establishment and leaf size were significantly higher in the *atnhx1* mutant than in the wild type [21]. Furthermore, the leaf epidermal cells of the mature *atnhx1* mutant were much smaller than those of the wild type at an equivalent developmental stage (i.e., equivalent age and position). It should be noted that this reduction in leaf cell size completely disappeared when *AtNHX1* was overexpressed in the *atnhx1* mutant. Vacuoles isolated from the leaves of the *atnhx1* mutant showed almost no Na^+/H^+ and K^+/H^+ activities despite the other five isoform genes being intact, suggesting that AtNHX1 is the predominant isoform in *Arabidopsis* leaves. These results indicate that AtNHX1 is also involved in leaf cell expansion, probably via regulating cation accumulation in the vacuole, which plays a central role in providing turgor necessary for cell expansion. Interestingly, the transcriptional profiling of wild type and *atnhx1* revealed that the loss of a functional *AtNHX1* had a large impact on the expression of other genes involved in ion homeostasis, vesicular trafficking, and protein targeting, suggesting that AtNHX1 has important roles in these processes [45]. The exact mechanisms leading to these alterations in gene expression are unknown at present. However, these results might indicate that cation and pH homeostasis mediated by NHX isoforms plays key roles in a number of cellular processes in addition to stress tolerance.

The physiological role of another NHX isoform, tomato *LeNHX2*, has also been studied by RNAi-mediated gene silencing, and it was found that the degree of gene silencing showed a strong correlation with plant growth and development under normal growth conditions: strongly silenced T0 lines showed poor growth compared to nontransgenic plants and also failed to produce fruits or seeds [31]. Furthermore, growth analysis with moderately silenced plants under saline conditions revealed that silenced plants were more susceptible to Na^+ compared to nontransgenic plants [31]. Although the exact mechanisms behind these phenotypes remain elusive, these results indicate the physiological significance of *LeNHX2* in the growth and development of tomato plants.

23.2.3
Transgenic Approaches to Increasing Salinity Tolerance Using NHX Genes

It has been shown that overexpression of *NHX* isoforms using constitutive promoters increases salinity tolerance of the host plant in all cases except one [46]. The first successful case reported by Apse *et al.* [25] demonstrated that overexpression of *AtNHX1* markedly increased salinity tolerance in *Arabidopsis*. The transgenic plant also showed increased vacuolar Na^+/H^+ antiport activity and cellular Na^+ sequestration, indicating that overexpression of *AtNHX1* increased the plant's ability to detoxify cellular Na^+ by compartmentation into the vacuole [25]. Overexpression of *AtNHX1* has been successfully employed to increase salt tolerance in tomato [47] and *Brassica napus* [48] without affecting their crop qualities. Enhancement of salt tolerance by overexpression of the *AtNHX1* gene has also been observed in other plant species including wheat (*Triticum aestivum* L.) [49], buckwheat (*Fagopyrum*

esculentum) [50], tall fescue (*Festuca arundinacea*) [51], and cotton (*Gossypium hirsutum* L.) [52]. In addition, *AtNHX3* and *AtNHX5* have been shown to increase salt tolerance when overexpressed in sugar beet (*Beta vulgaris*) [53] and Torenia (*Torenia fournieri*) [54], respectively. NHX isoforms from other plant species have also been utilized to increase salt tolerance through overexpression, either in the plant species of their origin or in heterologous host species. For example, enhanced salt tolerance in rice has been successfully achieved by introducing an additional copy of the endogenous *OsNHX1* gene [17], as well as several *NHX* isoform genes from halophytic species, namely, *Chenopodium glaucum* [55], *Pennisetum glaucum* [56], and *Atriplex gmelini* [57]. In addition, it has been shown that the overexpression of the *MdNHX1* gene from the salt-tolerant rootstock of apple (*Malus* × *domestica* Borkh.) conferred Na^+ tolerance to a salt-sensitive variety of apple [58]. These observations illustrate the effectiveness of the overexpression of *NHX* isoforms to produce salt-tolerant crops.

It should be noted, however, that there are some arguments regarding the mechanisms by which these transgenic plants circumvent Na^+ cytotoxicity. Although most of the above transgenic plants showed increased accumulation of Na^+ compared to their parental plants under saline conditions, there were some cases where transgenics exhibited no significant increase in Na^+ content, even though they did display increased salinity tolerance [31, 59]. In one such case, an *Arabidopsis* plant overexpressing *LeNHX2* showed increased K^+ accumulation, while Na^+ accumulation was less than that of the wild type [31]. Increased K^+ content was also observed in sugar beet overexpressing *AtNHX3* [53]. These results might suggest that the acquisition of salt tolerance by *NHX* overexpression may be attributed to, at least in some cases, the alteration of cellular K^+ homeostasis rather than increased compartmentation of Na^+ into the vacuole.

23.3 HKT Transporters

23.3.1 Overview

The first HKT-type transporter was identified in wheat (*T. aestivum*; TaHKT2;1, originally named HKT1). The nomenclature "HKT" is based on the initial description of the transporter's high affinity for K^+ transport (*h*igh-affinity K^+ *t*ransporter) [60]. However, subsequent detailed biophysical transport analyses revealed that TaHKT2;1 shows unique selectivity for Na^+, such that TaHKT2;1 mediates high-affinity Na^+/K^+ symport when the extracellular concentration of K^+ is higher than Na^+, but low-affinity Na^+-selective uniport is preferred when the Na^+ concentration far exceeds that of K^+ [61, 62]. HKT transporters have been isolated from a wide variety of plant species to date, including the halophyte *M. crystallinum* [63], and the ion selectivity of those HKT transporters has been well characterized, indicating that HKT transporters in general mediate robust Na^+ transport [9, 10].

Several plant HKT transporters including AtHKT1;1 from *Arabidopsis* have been shown to exhibit very poor K^+ permeability, preferring Na^+-selective uniport in eukaryotic heterologous cells [64–66]. Experimental approaches to determine the membrane structure of AtHKT1;1 have provided evidence for a four transmembrane–pore–transmembrane topology model [67], which is conserved in the K^+-conducting pore region of HKT/Trk/Ktr transporters as well as the K^+ channels of prokaryotic and eukaryotic cells [68, 69]. Heterologous expression studies of the Na^+-selective transporters *AtHKT1;1* and *OsHKT2;1*, and the K^+-transporting *TaHKT2;1* and *OsHKT2;2*, in combination with domain swapping and site-directed mutagenesis have revealed that a glycine residue (G) at the filter position in four putative p-loops is critical for robust K^+ permeability and more Na^+-selective HKT transporters retain a serine residue (S) at the filter position of the first pore region (SGGG type) in contrast to K^+-transporting HKT transporters that conserve the glycine residues in all four pore regions (GGGG type) [70]. Moreover, ion transport analyses of the bacterial KtrAB K^+ transporter from *Vibrio alginolyticus* have demonstrated that the glycines at the four filter positions are essential for robust K^+ permeability [71]. Together, these analyses support the p-loop hypothesis put forward for the HKT/Trk/Ktr transporters [68, 69]. Phylogenetic analysis of various HKT amino acid sequences has indicated the existence of at least two major subfamilies, namely, class I and class II [9, 10, 72]. One of the characteristics of class I HKT transporters is higher selectivity for Na^+ than other cations, which contrasts with class II HKT transporters that show robust permeability for both Na^+ and K^+. Differences in cation specificity between class I and class II HKT transporters show a close correlation with the glycine residues at the filter positions in the pore regions. Among the amino acid sequences of HKT transporters that are publicly available thus far, the filter positions of all class I HKT transporters are SGGG, as in AtHKT1;1 and OsHKT2;1 [65, 70], except for VvHKT1;2 from *V. vinifera*, which has the aspartic acid residue (D) in the second p-loop (SDGG type) [10]. Conversely, all class II HKT transporters, except for OsHKT2;1, conserve glycine residues at the filter positions, as in the cases of TaHKT2;1 and OsHKT2;2 (GGGG) [9, 10]. OsHKT2;1 is a unique class II transporter that exhibits class I transporter-like features, SGGG-type filter composition, and more Na^+-selective transport [65, 70]. Note that independent analyses have indicated that OsHKT2;1 shows broader selectivity to alkali monovalent cations in heterologous cells [66, 73]. More recent reports, however, have demonstrated that the Na^+-selective transport by OsHKT2;1 and Na^+/K^+ symport by OsHKT2;2 observed in heterologous cells [65, 70, 74] are consistently reproduced in the homologous plant cell system of cultured tobacco BY2 cells (*Nicotiana tabacum* L. cv Bright Yellow 2 cells) [75]. It has been shown that SGGG-type HKT transporters from *Eucalyptus camaldulensis* and *M. crystallinum* are permeable to K^+ when expressed in heterologous systems [63, 76]. Conserved positively charged residues and negatively charged residues have been identified as residues essential for the function of *Synechocystis* Ktr transporters and plant HKT transporters [77–79]. In particular, the electrostatic interaction present in HKT/Trk/Ktr transporters is proposed to be one of the determinants for ion conductivity resulting in the difference between ion channels and transporters [77, 80]. Several

other residues that are important for ion conductivity have also been found in the *Vibrio* Ktr transporter [81, 82].

23.3.2 Physiological Roles of HKTs

A. thaliana plants retains a single *HKT* gene, *AtHKT1;1*, in the genome [64]. Given that genetic suppressor screening using *sos3* (*salt overly sensitive 3*) mutant plants revealed that *AtHKT1;1* gene disruption rescues salt hypersensitivity of *sos3* plants, AtHKT1;1 was initially proposed to be a major pathway for Na^+ entry into roots during salinity stress [83]. Several subsequent reports, however, demonstrated that independent *athkt1;1* mutant lines displayed hypersensitivity to Na^+, accompanied by leaf chlorosis due to overaccumulation of Na^+ in the aerial organs, while maintaining lower root Na^+ concentrations compared to wild-type plants [84–89]. These reports proposed an essential physiological function of AtHKT1;1 in the salinity resistance mechanism in stelar cells in contrast to the proposed function from the *sos3* suppressor screening [83]. Berthomieu *et al.* proposed a "Na^+ recirculation" model, where AtHKT1;1 loads Na^+ into the phloem and Na^+ ions are transferred from shoot to root via the downward phloem stream, thereby preventing overaccumulation of Na^+ in the shoots (Figure 23.1) [85]. On the other hand, since localization of AtHKT1;1 has been observed at the plasma membrane in xylem parenchyma cells, a Na^+ unloading model, which similarly prevents overaccumulation of Na^+ in shoots, especially in leaves, was proposed (Figure 23.1) [84, 87–89]. Note that the role of AtHKT1;1 in the phloem needs to be further investigated as independent laboratories have drawn different conclusions; a significant effect of *AtHKT1;1* mutations on phloem sap under salt stress was found in one study [87], similar to the findings of Berthomieu *et al.* [85], but Na^+ recirculation was concluded not to occur based on $^{22}Na^+$ flux measurements in another study [89]. Further investigation will be needed to figure out the precise physiological function of AtHKT1;1 in stelar cells as a positive salinity resistance determinant. The discrepancies in the proposed functions of AtHKT1;1 *in vivo*, derived from the studies of *sos3 athkt1;1* double mutants and *athkt1;1* single mutants, have not yet been completely reconciled. However, one possible explanation has been proposed, in which the effect of *AtHKT1;1* mutations becomes predominant under higher Ca^{2+} conditions in *sos3 athkt1;1* double mutant plants, such that the plants display *athkt1;1*-derived salt hypersensitivity that suppresses *sos3*-mediated phenotypes [88].

An interesting indirect relationship between AtHKT1;1-mediated Na^+ removal and K^+ homeostasis across xylem vessels has been proposed in the xylem Na^+ unloading model [87]. AtHKT1;1-mediated Na^+ removal into xylem parenchyma cells would cause depolarization of the cells, which in turn triggers K^+ loading into xylem vessels, resulting in maintenance of high K^+/Na^+ ratios in the leaves. This working hypothesis is consistent with similar HKT transporter-mediated mechanisms in crop plants, which are described below (for details, see Refs [9, 10]).

Xylem Na^+ unloading by class I HKT transporters has also been demonstrated to occur in the monocot crop plants rice and wheat. The *SKC1* locus, one of the major

QTL for salt tolerance identified by crossing a salt-tolerant rice cultivar Nona Bokra with a salt-sensitive cultivar Koshihikari, has been found to control Na^+/K^+ content in the xylem sap, and it encodes a class I HKT transporter (OsHKT1;5) [90]. A comparison of *OsHKT1;5* sequences highlighted several point mutations between Nona Bokra-derived and Koshihikari-derived *OsHKT1;5* transporter genes, which were eventually found to be a cause of the difference in the Na^+ transport activity of the two OsHKT1;5 transporters such that Nona Bokra-derived OsHKT1;5 exhibits significantly higher Na^+ transport activity. Moreover, the Nona Bokra-derived OsHKT1;5 was shown to maintain lower Na^+ but higher K^+ contents in the xylem sap during salinity stress, indicating that Nona Bokra plants have the ability to retrieve more Na^+ from xylem vessels under salt stress [90].

Several genetic loci related to salinity tolerance have been found in wheat species, namely, the *Kna1* locus in *T. aestivum* [91] and the *Nax1* and *Nax2* loci in *T. monococcum* [92, 93], which have been shown to be involved in the maintenance of a high K^+/Na^+ ratio in leaves. The *Nax1* and *Nax2* loci were shown to play important roles in preventing root-to-shoot Na^+ transport by excluding Na^+ from the xylem [94]. Interestingly, the *Nax1* locus has been demonstrated to be a leaf-located mechanism controlling the Na^+ content of leaves by excluding Na^+ from the xylem of the leaf sheath, thus preventing Na^+ overaccumulation in the leaf blade. It has since been suggested that the *Nax1* locus encodes the TmHKT1;4 transporter, a class I HKT transporter [95]. In addition, the *Nax2* and *Kna1* loci are proposed to harbor the same gene, *HKT1;5* (*TmHKT1;5* and *TaHKT1;5*, respectively), which also encodes a class I transporter [96]. Together with the results from analyses of the *AtHKT1;1* gene, these results indicate that xylem Na^+ unloading is a common essential mechanism of salinity tolerance in these plants. Genetic studies of wheat class I HKT transporters implies that both HKT1;4 and HKT1;5 transporters might be necessary to exert complete salinity resistance in crop plants [95]. Studies of the molecular and physiological functions of HKT1;4, including OsHKT1;4 from rice, will be important to fully understand class I HKT transporter-mediated salinity resistance in plants.

Physiological functions of the class II HKT transporters have been less reported compared to class I HKT transporters. Several independent studies have demonstrated that mRNA accumulation of class II *HKT* genes in rice, wheat, and barley, including *OsHKT2;1* and *TaHKT2;1*, is induced under K^+-limiting conditions [65, 74, 97], which implies an essential role for these genes in K^+ homeostasis. In addition, the *TaHKT2;1* gene was found to be expressed in root cortical cells [60]. However, given that the *TaHKT2;1* gene knockdown wheat plants were shown to exhibit reduced root Na^+ influx and increased Na^+ sensitivity without showing any significant effect on K^+ uptake, TaHKT2;1 has been suggested to play a major role in Na^+ uptake in K^+-starved roots with a lesser contribution to K^+ uptake [98]. The K^+ uptake system in plants is suggested to be composed of multiple pathways [99–101]. Another possibility that should therefore be considered is that other high-affinity K^+ transport systems would mask the effect of gene knockdown/knockout of K^+-transporting class II HKT transporters. Whether class II HKT transporters might contribute to high-affinity K^+ uptake in plants remains to be elucidated.

OsHKT2;1 is an atypical member of the class II HKT transporters that retains SGGG filter amino acids and more Na^+-selective transport, as in class I HKT transporters [65, 70, 74, 75]. It has been demonstrated that independent null mutations of the *OsHKT2;1* gene cause a reduction in the shoot biomass of the rice mutant lines compared to wild-type plants under conditions with low Na^+ and no added K^+ [102]. Together with evidence for the lack of major Na^+ absorption ability in roots of K^+-starved *oshkt2;1* mutant plants, the OsHKT2;1 transporter has been assigned to play an indispensable role in K^+ starvation-induced nutritional Na^+ uptake and distribution in rice plants to maintain growth under low K^+ availability (Figure 23.1) [102].

In the japonica rice cultivar Nipponbare, only two GGGG-type class II OsHKT transporters, OsHKT2;3 and OsHKT2;4, were found to exist [74]. OsHKT2;3 and OsHKT2;4 are highly identical proteins (more than 93% identity at the amino acid sequence level). OsHKT2;4 has recently been found to display broad permeability to not only K^+ and Na^+ but also divalent cations including Ca^{2+} and Mg^{2+} [103]. Together with the evidence of the root hair plasma membrane localization of OsHKT2;4, a novel physiological role for OsHKT2;4 in Ca^{2+}-linked processes including Ca^{2+} uptake has been proposed [103]. However, evidence for the role of OsHKT2;4 in Ca^{2+} homeostasis *in vivo* in rice plants (e.g., using the *oshkt2;4* rice mutant) is required to confirm this hypothesis.

23.3.3
Transgenic Approaches to Increasing Salinity Tolerance Using Class I HKT Genes

Since the function of known class I HKT transporters is uptake of Na^+ into the cell, ectopic overexpression of this class of transporter genes using constitutive promoters might not be a recommended approach for improving salt tolerance in plants. In fact, overexpression of *AtHKT1;1* using the 35S promoter has been shown to lead to an increase in Na^+ susceptibility due to Na^+ overaccumulation in shoots [104]. Recently, Møller *et al.* demonstrated that the targeted expression of the *AtHKT1;1* gene in root stelar cells (including xylem parenchyma cells) using an enhancer trap system successfully increased Na^+ tolerance of *Arabidopsis* plants [104]. Patch clamp analysis revealed that the Na^+ influx capacities of transgenic root cells were markedly increased. Moreover, the root-to-shoot transfer of Na^+ was significantly decreased in transgenic plants (determined using $^{22}Na^+$ tracer), which is consistent with the finding that the Na^+ content in transgenic plants was lower in shoots but higher in roots compared to control plants [104]. Interestingly, the highest Na^+ accumulation was found in root cortical cells with minor accumulation in stelar cells, suggesting a radial movement of Na^+. Note that cortical cells are highly vacuolated and considered to have a large capacity to sequester Na^+, probably via vacuolar $Na^+(K^+)/H^+$ antiporters (for details, see Section 23.2). These results suggest that overexpression of AtHKT1;1 in root stelar cells enhanced retrieval of Na^+ from the xylem stream to stelar cells, which indirectly enhanced Na^+ sequestration in the vacuoles of cortical cells, resulting in protection of shoots from Na^+ overaccumulation [104]. Similar tissue-specific expression systems have been utilized to express *AtHKT1;1* in root

cortical/epidermal cells of *Arabidopsis* and cortical cells of rice. Interestingly, both attempts increased salt tolerance in the transgenic plants [105]. Overexpression of *AtHKT1;1* in root cortical cells enhanced the expression level of endogenous class I *HKT* genes (*AtHKT1;1* and *OsHKT1;5* for *Arabidopsis* and rice, respectively), thereby increasing Na^+ retrieval from the xylem and sequestration to the vacuole in cortical cells simultaneously [105]. Together, these tissue-specific *AtHKT1;1* expression analyses have indicated that enhancing the activity for Na^+ reabsorption from the xylem vessels is a powerful approach for increasing plant salinity tolerance.

23.4 SOS1 Transporters

23.4.1 Overview

In addition to compartmentation of Na^+ in the intracellular compartments, exclusion of Na^+ from the cytosol across the plasma membrane is also an important mechanism to alleviate cellular Na^+ toxicity in plants [1]. Earlier studies demonstrated the presence of Na^+/H^+ antiport activity at the plasma membrane in a number of plant species, including tomato [106, 107], barley [108], wheat [109], and red beet [110], and this activity was presumed to be responsible for Na^+ efflux from the cell [1]. The SOS1 transporter is one of the best-characterized class of transporters that show Na^+ efflux activity in land plants. The *SOS1* gene was first identified by genetic screening as an essential gene that contributes to salt tolerance in *Arabidopsis* [111]. Genetic mapping and positional cloning revealed that the *Arabidopsis SOS1* gene (also named *AtNHX7*, but hereafter referred to as *AtSOS1*) encodes a large putative membrane protein (127 kDa, 1146 aa) that shares substantial similarities to several known Na^+/H^+ antiporters, including the mammalian NHE1 and the bacterial NhaA [112]. Detailed phylogenetic analysis with other cation/H^+ antiporters showed that AtSOS1 is categorized into the CPA1 family along with NHEs and NHXs, although it is more closely related to bacterial NhaP antiporters [15, 112, 113]. An analysis of the subcellular distribution by overexpressing AtSOS1-GFP fusion proteins in *Arabidopsis* plants indicated that AtSOS1 is localized in the plasma membrane, which was further supported by results of subcellular fractionation [114]. Promoter–GUS analysis has revealed that the *AtSOS1* transcript is predominantly found in the epidermal tissues of root tips and xylem parenchyma cells of roots, stems, and leaves [115]. *AtSOS1* mRNA expression is specifically induced in shoots and roots by salt treatment, but not by cold or ABA treatment [112]. Interestingly, the induction of *AtSOS1* expression appears to be under the control of other *AtSOS* genes, namely, *AtSOS2* (encoding a serine/threonine protein kinase) and *AtSOS3* (encoding a calcium binding protein) since induction of *AtSOS1* expression by salt treatment in *atsos2* and *atsos3* mutant plants is partially and almost completely abolished, respectively [112]. In addition, it seems that the mRNA stability of *AtSOS1* is also regulated by salinity stress since the amount of *AtSOS1*

transcripts in transgenic *Arabidopsis* plants harboring an additional copy of the *AtSOS1* ORF under the control of a constitutive promoter was significantly increased under saline conditions compared to nonstress conditions [116]. Hydropathy prediction indicated that the AtSOS1 protein consists of 12 putative transmembrane segments and a long C-terminal hydrophilic tail [112], although detailed structural properties of AtSOS1 remain to be studied.

It has been shown that AtSOS1 is able to complement the Na^+ sensitivity of yeast mutants lacking functional genes for plasma membrane Na^+-ATPases (Ena1–4) and two Na^+/H^+ antiporters localized to the plasma membrane (Nha1) or prevacuole (Nhx1) [115]. Interestingly, phenotypes not directly related to Na^+ efflux (i.e., sensitivity to high external K^+ of the *nha1* mutant and hygromycin B sensitivity of the *nhx1* mutant) were not rescued by the expression of *AtSOS1*, suggesting that AtSOS1 is a Na^+-specific transporter [115]. Transport activity of endogenous AtSOS1 was demonstrated by comparing the Na^+/H^+ antiport activities of highly purified plasma membrane vesicles from the leaves of wild-type and *atsos1* mutant *Arabidopsis* plants pretreated with NaCl; the Na^+/H^+ antiport activity of plasma membrane vesicles of the *atsos1* mutant was significantly lower than that of wild type [117]. Further study using a similar approach characterized AtSOS1 both as a low-affinity Na^+/H^+ antiporter (apparent $K_m = 22.8$ mM) that does not accept K^+ or Li^+ as substrates and as an electroneutral transporter [114].

It should be noted that AtSOS1 requires AtSOS2 and AtSOS3 to be fully activated since AtSOS1 activities of plasma membrane vesicles from *atsos2* and *atsos3* mutant plants were similar to the *atsos1* mutant [117]. Moreover, the addition of constitutively active AtSOS2 proteins was found to significantly increase Na^+/H^+ antiport activities [117]. A separate study using a yeast heterologous expression system showed that AtSOS1 could be directly phosphorylated by AtSOS2 kinase [118], and this phosphorylation by AtSOS2 is enhanced when AtSOS3, which is known to form a complex with AtSOS2 [119], coexisted. Furthermore, it has been shown that AtSOS3 is able to recruit AtSOS2 protein to the plasma membrane [118]. On the basis of these results, AtSOS1 is known to be a part of the so-called "SOS pathway," in which AtSOS3 delivers otherwise cytosolic AtSOS2 kinase to the plasma membrane, where AtSOS2 activates its target AtSOS1 by phosphorylation [114, 118].

SOS1 orthologues have been isolated from several plants, including rice [120], wheat [121], tomato [122], and halophytic *Thellungiella salsuginea* (formerly *T. halophila*) [123]. All these SOS1 orthologues are shown to at least have the ability to rescue mutant yeast or *Escherichia coli* that are deficient in Na^+ export, and recent RNAi studies in tomato and *T. salsuginea* have indicated that SOS1 plays major roles in salt tolerance in these plant species [122, 124–126] (see below).

In addition to the above orthologues, a *SOS1*-like gene, *AtNHX8*, has been identified in *Arabidopsis*. AtNHX8 is the closest homologue of AtSOS1 and shares more than 88% similarity in the hydrophobic region but lacks approximately half the hydrophilic tail at the C-terminus (83 kDa, 756 aa). Detailed characterization using a heterologous yeast expression system and *atnhx8* null mutant plants indicate that despite its high sequence similarity to AtSOS1, the *AtNHX8* gene likely encodes a Li^+/H^+ antiporter rather than a Na^+/H^+ antiporter [127].

23.4.2 Physiological Roles of SOS1

The physiological significance of AtSOS1 in saline environments has been well established in *Arabidopsis*, as *atsos1* null alleles exhibit intense salt sensitivity [111, 112]. The *atsos1* mutant has been shown to accumulate a higher amount of Na^+ compared to wild-type plants under severe salt stress, with the difference in Na^+ accumulation being more apparent in roots than in shoots [115]. Given that AtSOS1 expression is found predominantly in the epidermis of *Arabidopsis* root tips and increases with Na^+ stress, and that its proposed function is as a Na^+/H^+ antiporter at the plasma membrane (see above), it has been suggested that AtSOS1 plays a critical role in Na^+ efflux from root cells, which limits net Na^+ entry (Figure 23.1) [115, 117]. The results from proton flux measurements have indicated that SOS1 is involved in Na^+-dependent H^+ influx at the meristem zone of *Arabidopsis* roots, supporting this view [128].

It has been suggested that AtSOS1 is also involved in the long-distance transport of Na^+. The Na^+ accumulation in shoots of *atsos1* mutant plants was less than that of wild-type plants under moderate salt stress when cultivated in hydroponic or high humidity conditions [116, 129]. This observation, together with the xylem parenchyma cell-specific expression pattern of the *AtSOS1* transcript [116], prompted the suggestion that AtSOS1 might play a role in loading of Na^+ into the xylem stream under moderate salt stress and no or low transpiration (Figure 23.1), although its physiological significance in *Arabidopsis* is yet to be determined.

A recent RNAi study in tomato (*Solanum lycopersicum*) [122] has revealed another role for SOS1 transporters under salinity stress. The *SlSOS1*-silenced line showed increased Na^+ content in roots and leaves compared to wild type under salt stress, consistent with the suggested role for AtSOS1 in *Arabidopsis*, but stems of silenced plants accumulated less Na^+ compared to the wild type [122, 124]. These results indicated that SlSOS1 plays a key role in the retention of Na^+ in the stem in tomato. Although direct evidence is not available at this point, the authors hypothesized that SlSOS1 might function in Na^+ efflux from the leaf cells to the xylem stream to protect the photosynthetic organs (Figure 23.1), and the higher leaf/root Na^+ and lower stem Na^+ concentrations observed in silenced plants were the consequence of a loss of this process, while the xylem unloading of Na^+ mediated by HKT1 remained intact [122, 124].

An RNAi suppression study has also revealed a critical role of SOS1 in *T. salsuginea*, which is an extremely salt-tolerant species closely related to *Arabidopsis*. The silenced plants exhibited faster Na^+ accumulation under saline conditions and slower Na^+ efflux after removal of salt, indicating that SOS1 plays a similar role in Na^+ efflux in roots as observed in *Arabidopsis* [125]. Propidium iodide staining detected cell death in the silenced plants, initially occurring at the elongating zone of the roots and expanding to older tissues as the salt stress continued, indicating that SOS1 protects these susceptible regions from Na^+ accumulation by excluding it at the root tip, where SOS1 is most abundantly expressed [125]. The expression level of *SOS1* in wild-type *T. salsuginea* was reported to be 10 times higher than that of *Arabidopsis* under saline conditions [125]. Furthermore, the suppression of *SOS1* expression

resulted in a loss of its halophytic nature, as root growth inhibition and partial chlorosis were observed at 200 mM NaCl, conditions under which *T. salsuginea* plants can usually grow without symptoms but are toxic to *Arabidopsis* [125]. These results indicate that SOS1 plays a key role in the halophytism of *T. salsuginea* [125].

Besides its critical roles in salt tolerance, SOS1 appears to be involved in several other cellular processes that do not seem to be directly related to Na^+ transport. A recent study using fluorescent probes reported that the loss of a functional *AtSOS1* gene resulted in altered pH homeostasis, both in the cytosol and in the vacuole of root cells [130]. Since AtSOS1 expression has not been found in the tonoplast membrane, the change in the vacuolar pH homeostasis is likely to be an indirect consequence of the alteration in plasma membrane H^+ flux, which has also been demonstrated by the electrophysiological study of *atsos1* [131]. In addition, fragmentation of the vacuoles and defects in endocytosis under salinity stress have been observed in *atsos1* mutants [130]. This is probably related to the altered cellular pH homeostasis, as modulations of H^+-translocating protein activities often affect intracellular membrane trafficking [113, 132–134]. In addition, SOS1 appears to play a role in protecting the root plasma membrane K^+ uptake under salinity stress since inhibition of root K^+ permeability under moderate salinity stress and growth under K^+-limiting conditions were observed in *atsos1* but not in wild-type plants [135].

23.4.3
Transgenic Approaches to Increasing Salinity Tolerance Using SOS1 Genes

It has been shown that ectopic expression of *AtSOS1* using the 35S promoter in *Arabidopsis* confers resistance to salinity stress, as less growth inhibition was observed in transgenic plants compared to nontransgenic plants under salinity stress [46, 116]. Transgenic plants and undifferentiated calli also exhibited less accumulation of Na^+, suggesting that enhancement of salinity tolerance by overexpression of AtSOS1 could be due to the limitation of Na^+ accumulation, which occurs through increased Na^+ efflux via AtSOS1 [116]. Similar growth improvements under saline conditions have also been observed in *Arabidopsis* plants that overexpress the *T. salsuginea SOS1* [125] or *OsSOS1* [120]. These results indicate that overexpression of a *SOS1* gene could be a valid approach to improve the salinity tolerance of crop plants.

23.5
Other Molecules that are Potentially Useful for Improving Salt Tolerance

23.5.1
HAK/KUP/KT Transporters

K^+ is an essential macronutrient for growth and the most abundant cation in plant cells. K^+ ions play vital roles in many homeostatic processes including osmoregulation, maintenance of membrane potential, and photosynthesis [100, 136]. K^+ is also an important factor in determining salt tolerance since K^+ absorption is

competitively blocked by Na^+ and K^+ alleviates the toxic effects of Na^+ [9–11]. Therefore, one potential strategy to engineer salt-tolerant plants is to enhance K^+ acquisition during salinity stress. Recently, the relatively Na^+-insensitive K^+ transport function of the OsHAK5 transporter from rice was identified in heterologous *E. coli* and yeast cells, and attempts have been made to utilize this as a tool to increase salinity tolerance in plant cells [137]. OsHAK transporters are members of the HAK/KUP/KT transporter family that constitutes a major K^+ transporter family in plants [100]. Constitutive expression of OsHAK5 in cultured tobacco BY2 cells was found to increase K^+ accumulation but decrease Na^+ accumulation during salt stress compared to control cells, conferring increased salt tolerance to the BY2 cells [137]. The study suggested that enhanced K^+ acquisition would also lead to a decrease in Na^+ accumulation, presumably due to depolarization of the membrane potential through increased K^+ uptake. These results indicate that the plasma membrane localized Na^+-insensitive K^+ transporters could be a tool to produce salt-tolerant plants. Further evaluation of the effect of OsHAK5 expression on plant salt tolerance will be needed at the individual plant level, in addition to identification of similar Na^+-insensitive K^+ transporters/channels.

23.5.2 ENA1/PMR2A

Fungi, including budding yeast (*Saccharomyces cerevisiae*), have a plasma membrane Na^+-ATPase that mediates Na^+ efflux as a major means of resisting salt stress [138, 139]. Although *S. cerevisiae* retains a tandem array of P-type Na^+-ATPase genes, only the *ENA1/PMR2A* gene encoding Ena1p was found to be strongly induced by salt stress [140, 141]. Ena1p tagged with the triple hemagglutinin tag (Ena1p-3HA) was expressed in cultured tobacco BY2 cells [142]. Ena1p-3HA expressing BY2 cells showed increased NaCl and LiCl tolerance by maintaining lower levels of Na^+ and Li^+ than control cells under salt stress conditions [142]. These results indicate that Ena1p is a candidate for increasing plant salt tolerance. ENA1-like P-type ATPases have been found in a primitive moss *Physcomitrella patens* [143]. PpENA1 was demonstrated to be a Na^+ pump and was suggested to play an important role in extruding Na^+ under salt stress as a mechanism for the moss to resist Na^+ toxicity [143]. It seems likely that such Na^+ pump activity was lost in flowering plants during evolution, and it might suggest that the Na^+ pump system is a not favorable mechanism due to the energy-consuming nature of the Na^+ pump. Further study using Ena1p expressing transgenic plants in combination with tissue-specific promoters will be necessary to evaluate the utility of the Na^+-ATPase for increasing plant salt tolerance.

23.6 Conclusions

Merely two decades ago, there was no evidence for Na^+ influx/efflux or a Na^+ circulation system in plants because of Na^+ being a nonessential element for plant

growth and development. Since the cloning of *HKT*, *SOS1*, *NHX*, and *HAK/KUP/KT* transporter genes from higher plants, numerous Na^+ transporters have been characterized. A large number of studies have provided evidence that these transporters play crucial roles in plants, and these studies have revealed the detailed mechanisms involved in the removal of Na^+ from cells to prevent cytotoxicity. Moreover, these studies have shed light on the potential for developing engineered elite traits using ion transporter genes. Further progress in identifying and characterizing the transporters involved in Na^+/K^+ homeostasis can be expected to increase our understanding of the mechanisms underlying this process in addition to aiding the development of tools for improving salinity tolerance in plants.

References

1 Blumwald, E. (2000) *Curr. Opin. Cell Biol.*, **12**, 431–434.
2 Blumwald, E., Aharon, G.S., and Apse, M.P. (2000) *Biochim. Biophys. Acta*, **1465**, 140–151.
3 Munns, R. and Tester, M. (2008) *Annu. Rev. Plant Biol.*, **59**, 651–681.
4 Hall, J.L. and Flowers, T.J. (1973) *Planta*, **110**, 361–368.
5 Flowers, T.J. and Läuchli, A. (1983) Sodium versus potassium: substitution and compartmentation, in *Inorganic Plant Nutrition*, New Series, vol. **15B**, Encyclopedia of Plant Physiology (eds A. Läuchli and R.A. Bieleski), Springer, Berlin, pp. 651–681.
6 Murguía, J.R., Bellés, J.M., and Serrano, R. (1995) *Science*, **267**, 232–234.
7 Tsugane, K., Kobayashi, K., Niwa, Y., *et al.* (1999) *Plant Cell*, **11**, 1195–1206.
8 Tsunekawa, K., Shijuku, T., Hayashimoto, M., *et al.* (2009) *J. Biol. Chem.*, **284**, 16513–16521.
9 Horie, T., Hauser, F., and Schroeder, J.I. (2009) *Trends Plant Sci.*, **14**, 660–668.
10 Hauser, F. and Horie, T. (2010) *Plant Cell Environ.*, **33**, 552–565.
11 Uozumi, N. and Schroeder, J.I. (2010) Ion channels and plant stress: past, present and future, in *Ion Channels and Plant Stress Responses* (eds V. Demidchik and F. Maathuis), Springer, Berlin, pp. 1–22.
12 Gaxiola, R.A., Rao, R., Sherman, A., *et al.* (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 1480–1485.
13 Fukuda, A., Nakamura, A., and Tanaka, Y. (1999) *Biochim. Biophys. Acta.*, **1446**, 149–155.
14 Pardo, J.M., Cubero, B., Leidi, E.O., *et al.* (2006) *J. Exp. Bot.*, **57**, 1181–1199.
15 Rodoríguez-Rosales, M.P., Gálvez, F.J., Huertas, R., *et al.* (2009) *Plant Signal. Behav.*, **4**, 265–276.
16 Yamaguchi, T. and Blumwald, E. (2005) *Trends Plant Sci.*, **10**, 615–620.
17 Fukuda, A., Nakamura, A., Tagiri, A., *et al.* (2004) *Plant Cell Physiol.*, **45**, 146–159.
18 Aharon, G.S., Apse, M.P., Duan, S., *et al.* (2003) *Plant Soil*, **253**, 245–256.
19 Yokoi, S., Quintero, F.J., Cubero, B., *et al.* (2002) *Plant J.*, **30**, 529–539.
20 Yamaguchi, T., Fukada-Tanaka, S., Inagaki, Y., *et al.* (2001) *Plant Cell Physiol.*, **42**, 451–461.
21 Apse, M.P., Sottosanto, J.B., and Blumwald, E. (2003) *Plant J.*, **36**, 229–239.
22 Venema, K., Quintero, F.J., Pardo, J.M., *et al.* (2002) *J. Biol. Chem.*, **277**, 2413–2418.
23 Venema, K., Belver, A., Marín-Manzano, M.C., *et al.* (2003) *J. Biol. Chem.*, **278**, 22453–22459.
24 Säier, M.H., Jr., Tran, C.V., and Barabote, R.D. (2006) *Nucleic Acids Res.*, **34**, D181–D186
25 Apse, M.P., Aharon, G.S., Snedden, W.A., *et al.* (1999) *Science*, **285**, 1256–1258.
26 Hamada, A., Shono, M., Xia, T., *et al.* (2001) *Plant Mol. Biol.*, **46**, 35–42.
27 Xia, T., Apse, M.P., Aharon, G.S., *et al.* (2002) *Physiol. Plant.*, **116**, 206–212.

28 Yoshida, K., Kawachi, M., Mori, M., *et al.* (2004) *Plant Cell Physiol.*, **46**, 407–415.

29 Ohnishi, M., Fukada-Tanaka, S., Hoshino, A., *et al.* (2005) *Plant Cell Physiol.*, **46**, 259–267.

30 Li, W.Y., Wong, F.L., Tsai, S.N., *et al.* (2006) *Plant Cell Environ.*, **29**, 1122–1137.

31 Rodríguez-Rosales, M.P., Jiang, X.J., Gálvez, F.J., *et al.* (2008) *New Phytol.*, **179**, 366–377.

32 Bowers, K., Boaz, L.P., Patel, F.I., *et al.* (2000) *Mol. Biol. Cell.*, **11**, 4277–4294.

33 Chauhan, S., Forsthoefel, N., Ran, Y., *et al.* (2000) *Plant J.*, **24**, 511–522.

34 Yamaguchi, T., Apse, M.P., Shi, H., *et al.* (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 12510–12515.

35 Wakabayashi, S., Fafournoux, P., Sardet, C., *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2424–2428.

36 Putney, L.K., Denker, S.P., and Barber, D.L. (2002) *Annu. Rev. Pharmacol. Toxicol.*, **42**, 527–552.

37 Waditee, R., Hibino, T., Tanaka, Y., *et al.* (2001) *J. Biol. Chem.*, **276**, 36931–36938.

38 Yamaguchi, T., Aharon, G.S., Sottosato, J., *et al.* (2005) *Proc. Natl. Acad. Sci. USA*, **102**, 16107–16112.

39 Gruwel, M.L.H., Rauw, V.L., Loewen, M., *et al.* (2001) *Plant Sci.*, **160**, 785–794.

40 Okazaki, Y., Kikuyama, M., Hiramoto, Y., *et al.* (1996) *Plant Cell Environ.*, **19**, 569–576.

41 Sato, Y. and Sakaguchi, M. (2005) *J. Biochem. (Tokyo)*, **138**, 425–431.

42 Wakabayashi, S., Pang, T., Su, X., *et al.* (2000) *J. Biol. Chem.*, **275**, 7942–7949.

43 Shi, H. and Zhu, J.K. (2002) *Plant Mol. Biol.*, **50**, 543–550.

44 Hanana, M., Cagnac, O., Yamaguchi, T., *et al.* (2007) *Plant Cell Physiol.*, **48**, 804–811.

45 Sottosanto, J.B., Gelli, A., and Blumwald, E. (2004) *Plant J.*, **40**, 752–771.

46 Yang, Q., Chen, Z.Z., Zhou, X.F., *et al.* (2009) *Mol. Plant*, **2**, 22–31.

47 Zhang, H.X. and Blumwald, E. (2001) *Nat. Biotechnol.*, **19**, 765–768.

48 Zhang, H.X., Hodson, J.N., Williams, J.P., *et al.* (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 12832–12836.

49 Xue, Z.Y., Zhi, D.Y., Xue, G.P., *et al.* (2004) *Plant Sci.*, **167**, 849–859.

50 Chen, L.H., Zhang, B., and Xu, Z.Q. (2008) *Transgenic Res.*, **17**, 121–132.

51 Zhao, J., Zhi, D., Xue, Z., *et al.* (2007) *J. Plant Physiol.*, **164**, 1377–1383.

52 He, C., Yan, J., Shen, G., *et al.* (2005) *Plant Cell Physiol.*, **46**, 1848–1854.

53 Liu, H., Wang, Q., Yu, M., *et al.* (2008) *Plant Cell Environ.*, **31**, 1325–1334.

54 Shi, L.Y., Li, H.Q., Pan, X.P., *et al.* (2008) *Funct. Plant Biol.*, **35**, 185–192.

55 Li, J.Y., He, X.W., Xu, L., *et al.* (2007) *J. Zhejiang Univ. Sci. B*, **9**, 132–140.

56 Verma, D., Singla-Pareek, S.L., Rajagopal, D., *et al.* (2007) *J. Biosci.*, **32**, 621–628.

57 Ohta, M., Hayashi, Y., Nakashima, A., *et al.* (2002) *FEBS Lett.*, **532**, 279–282.

58 Li, Y., Zhang, Y., Feng, F., *et al.* (2010) *Plant Cell Tissue Organ Cult.*, **102**, 337–345.

59 Zhang, G.H., Su, Q., An, L.J., *et al.* (2008) *Plant Physiol. Biochem.*, **46**, 117–126.

60 Schachtmann, D.P. and Schroeder, J.I. (1994) *Nature*, **370**, 655–658.

61 Rubio, F., Gassmann, W., and Schroeder, J. (1995) *Science*, **270**, 1660–1663.

62 Gassmann, W., Rubio, F., and Schroeder, J.I. (1996) *Plant J.*, **10**, 869–882.

63 Su, H., Balderas, E., Vera-Estrella, R., *et al.* (2003) *Plant Mol. Biol.*, **52**, 967–980.

64 Uozumi, N., Kim, E.J., Rubio, F., *et al.* (2000) *Plant Physiol.*, **122**, 1249–1260.

65 Horie, T., Yoshida, K., Nakayama, H., *et al.* (2001) *Plant J.*, **27**, 129–138.

66 Jabnoune, M., Espeout, S., Mieulet, D., *et al.* (2009) *Plant Physiol.*, **150**, 1955–1971.

67 Kato, Y., Sakaguchi, M., Mori, Y., *et al.* (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 6488–6493.

68 Durell, S.R. and Guy, H.R. (1999) *Biophys. J.*, **77**, 789–807.

69 Durell, S.R., Hao, Y., Nakamura, T., *et al.* (1999) *Biophys. J.*, **77**, 775–788.

70 Mäser, P., Hosoo, Y., Goshima, S., *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 6428–6433.

71 Tholema, N., Von der Brüggen, M., Mäser, P., *et al.* (2005) *J. Biol. Chem.*, **280**, 41146–41154.

72 Platten, J.D., Cotsaftis, O., Berthomieu, P., *et al.* (2006) *Trends Plant Sci.*, **11**, 372–374.

73 Golldack, D., Su, H., Ouigley, F., *et al.* (2002) *Plant J.*, **34**, 1–14.

74 Garciadeblás, B., Senn, M., Bañuelos, M., *et al.* (2003) *Plant J.*, **34**, 788–801.

75 Yao, X., Horie, T., Xue, X., *et al.* (2010) *Plant Physiol.*, **152**, 341–355.

76 Fairbairn, D.J., Liw., W., Schachtman, D.P., *et al.* (2000) *Plant Mol. Biol.*, **43**, 515–525.

77 Kato, N., Akai, M., Zulkifli, L., *et al.* (2007) *Channels*, **1**, 161–171.

78 Zulkifli, L. and Uozumi, N. (2006) *J. Bacteriol.*, **188**, 7985–7987.

79 Zulkifli, L., Akai, M., Yoshikawa, A., *et al.* (2010) *J. Bacteriol.*, **192**, 5063–5070.

80 Uozumi, N. and Deryer, I. (2012) Structure–function correlates in plant ion channels, in *Comprehensive Biophysics Volume 6 - Channel Proteins* (ed. E. Egelman), Elsevier BV, Amsterdam, in press.

81 Hänelt, I., Löchte, S., Sundermann, L., *et al.* (2010) *J. Biol. Chem.*, **285**, 10318–10327.

82 Hänelt, I., Wunnicke, D., Müller-Trimbusch, M., *et al.* (2010) *J. Biol. Chem.*, **285**, 28210–28219.

83 Rus, A., Yokoi, S., Sharkhuu, A., *et al.* (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 14150–14155.

84 Mäser, P., Eckelman, B., Vaidyanathan, R., *et al.* (2002) *FEBS Lett.*, **531**, 157–161.

85 Berthomieu, P., Conéjéro, G., Nublat, A., *et al.* (2003) *EMBO J.*, **22**, 2004–2014.

86 Gong, J.I., Waner, D., Horie, T., *et al.* (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 15404–15409.

87 Sunarpi, Horie, T., Motoda, J., *et al.* (2005) *Plant J.*, **44** 928–938.

88 Horie, T., Horie, R., Chan, W.Y., *et al.* (2006) *Plant Cell Physiol.*, **47**, 622–633.

89 Davenport, R.J., Munoz-Mayor, A., Jha, D., *et al.* (2007) *Plant Cell Environ.*, **30**, 497–507.

90 Ren, Z.H., Gao, J.P., Li, L.G., *et al.* (2005) *Nat. Genet.*, **37**, 1141–1146.

91 Dubcovsky, J., Maris, G.S., Epstein, E., *et al.* (1996) *Theor. Appl. Genet.*, **92**, 448–454.

92 Munns, R., Rebetzke, G.J., Husain, S., *et al.* (2003) *Aust. J. Agric. Res.*, **54**, 627–635.

93 Lindsay, M.P., Lagudah, E.S., Hare, R.A., *et al.* (2004) *Funct. Plant Biol.*, **31**, 1105–1114.

94 James, R.A., Davenport, R.J., and Munns, R. (2006) *Plant Physiol.*, **142**, 1537–1547.

95 Huang, S., Spielmeyer, W., Lagudah, E.S., *et al.* (2006) *Plant Physiol.*, **142**, 1718–1727.

96 Byrt, C.S., Platten, J.D., Spielmeyer, W., *et al.* (2007) *Plant Physiol.*, **143**, 1918–1928.

97 Wang, T.B., Gassmann, W., Rubio, F., *et al.* (1998) *Plant Physiol.*, **118**, 651–659.

98 Laurie, S., Feeney, K.A., Maathuis, F.J., *et al.* (2002) *Plant J.*, **32**, 139–149.

99 Véry, A.A. and Sentenac, H. (2003) *Annu. Rev. Plant Biol.*, **54**, 575–603.

100 Gierth, M. and Mäser, P. (2007) *FEBS Lett.*, **581**, 2348–2356.

101 Lebaudy, A., Véry, A.A., and Sentenac, H. (2007) *FEBS Lett.*, **581**, 2357–2366.

102 Horie, T., Costa, A., Kim, T.H., *et al.* (2007) *EMBO J.*, **26**, 3003–3014.

103 Lan, W.Z., Wang, W., Wang, S.M., *et al.* (2010) *Proc. Natl. Acad. Sci. USA*, **107**, 7089–7094.

104 Møller, I.S., Gilliham, M., Jha, D., *et al.* (2009) *Plant Cell*, **21**, 2163–2178.

105 Plett, D., Safwat, G., Gilliham, M., *et al.* (2010) *PLoS One*, **5**, e12571.

106 Mennen, H., Jacoby, B., and Marschner, H. (1990) *J. Plant. Physiol.*, **137**, 180–183.

107 Wilson, C. and Shannon, M.C. (1995) *Plant Sci.*, **107**, 147–157.

108 Ratner, A. and Jacoby, B. (1976) *J. Exp. Bot.*, **27**, 843–852.

109 Allen, G.J., Jones, R.G.W., and Leigh, R.A. (1995) *Plant Cell Environ.*, **18**, 105–115.

110 Jacoby, B. and Teomi, S. (1988) *Plant Sci.*, **55**, 103–106.

111 Wu, S.J., Ding, L., and Zhu, J.K. (1996) *Plant Cell*, **8**, 617–627.

112 Shi, H., Ishitani, M., Kim, C., *et al.* (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 6896–6901.

113 Brett, C.L., Donowitz, M., and Rao, R. (2005) *Am. J. Physiol. Cell Physiol.*, **288**, C223–C239

114 Qiu, Q.S., Barkla, B.J., Vera-Estrella, R., *et al.* (2003) *Plant Physiol.*, **2003**, 1041–1052.

115 Shi, H., Quintero, F.J., Pardo, J.M., *et al.* (2002) *Plant Cell*, **14**, 465–477.

116 Shi, H., Lee, B.H., Wu, S.J., *et al.* (2003) *Nat. Biotechnol.*, **21**, 81–85.

117 Qiu, Q.S., Guo, Y., Dietrich, M.A., *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 8436–8441.

118 Quintero, F.J., Ohta, M., Shi, H., *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 9061–9066.

119 Halfter, U., Ishitani, M., and Zhu, J.K. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 3735–3740.

120 Martínez-Atienza, J., Jiang, X., Garciadeblas, B., *et al.* (2007) *Plant Physiol.*, **143**, 1001–1012.

121 Xu, H., Jiang, X., Zhan, K., *et al.* (2008) *Arch. Biochem. Biophys.*, **473**, 8–15.

122 Olías, R., Eljakaoui, Z., Li, J., *et al.* (2009) *Plant Cell Environ.*, **32**, 904–916

123 Vera-Estrella, R., Barkla, B.J., García-Ramírez, L., *et al.* (2005) *Plant Physiol.*, **139**, 1507–1517

124 Olías, R., Eljakaoui, Z., Pardo, J.M., *et al.* (2009) *Plant Signal. Behav.*, **4**, 973–976

125 Oh, D.H., Leidi, E., Zhang, Q., *et al.* (2009) *Plant Physiol.*, **151**, 210–222.

126 Oh, D.H., Zahir, A., Yun, D.J., *et al.* (2009) *Plant Signal. Behav.*, **4**, 1081–1083.

127 An, R., Chen, Q.J., Chai, M.F., *et al.* (2007) *Plant J.*, **49**, 718–728.

128 Guo, K.M., Babourina, O., and Rengel, Z. (2009) *Physiol. Plant.*, **137**, 155–165.

129 Ding, L. and Zhu, J. (1997) *Plant Physiol.*, **113**, 795–799.

130 Oh, D.H., Lee, S.Y., Bressan, R.A., *et al.* (2010) *J. Exp. Bot.*, **61**, 1205–1213.

131 Shabala, L., Cui, T.A., Newman, I.A., *et al.* (2005) *Planta*, **222**, 1041–1050.

132 Brett, C.L., Tukaye, D.N., Mukherjee, S., *et al.* (2005) *Mol. Biol. Cell*, **16**, 1396–1405.

133 Nakamura, N., Tanaka, S., Teko, Y., *et al.* (2005) *J. Biol. Chem.*, **280**, 1561–1572.

134 Li, J., Yang, H., Peer, W.A., *et al.* (2005) *Science*, **310**, 121–125.

135 Qi, Z. and Spalding, E.P. (2004) *Plant Physiol.*, **136**, 2548–2555.

136 Schroeder, J.I., Ward, J.M., and Gassmann, W. (1994) *Annu. Rev. Biophys. Biomol. Struct.*, **23**, 441–471.

137 Horie, T., Sugawara, M., Okada, T., *et al.* (2011) *J. Biosci. Bioeng.*, **111**, 346–356.

138 Haro, R., Garciadeblas, B., and Rodríguez-Navarro, A. (1991) *FEBS Lett.*, **291**, 189–191.

139 Prior, C., Potier, S., Souciet, J.L., *et al.* (1996) *FEBS Lett.*, **387**, 89–93.

140 Garciadeblas, B., Rubio, F., Quintero, F.J., *et al.* (1993) *Mol. Gen. Genet.*, **236**, 363–368.

141 Wieland, J., Nitsche, A.M., Strayle, J., *et al.* (1995) *EMBO J.*, **14**, 3870–3882.

142 Nakayama, H., Yoshida, K., and Shinmyo, A. (2004) *Biotechnol. Bioeng.*, **85**, 776–789.

143 Benito, B. and Rodríguez-Navarro, A. (2003) *Plant J.*, **36**, 382–389.

24
Piriformospora indica, A Root Endophytic Fungus, Enhances Abiotic Stress Tolerance of the Host Plant

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Piriformospora indica is an endophytic fungus that colonizes the roots of both monocot and dicot plants including members of the family Brassicaceae, which are nonhost for arbuscular mycorrhizal fungi (AMF) and can also be grown axenically. Like the AMF, *P. indica* was found to be involved in the enhancement of plant tolerance against abiotic stress. Growth promotion in plant is a characteristic effect of the fungal colonization, which can also be observed under the stress conditions. *P. indica* modulates the defense system and alters the metabolism to compensate the loss in photosynthesis and prevent oxidative damage caused by stress. Primarily, *P. indica* induces the defense system, especially the ascorbate–glutathione (ASH-GSH) cycle, and maintains a high antioxidative environment during salt and drought stress. *P. indica* also induces sevaral antioxidative enzymes during salt and drought stress that are involved in detoxification of reactive oxygen species (ROS) such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POD), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and so on. *P. indica* also increases the level of osmolytes such as polyamine and proline in response to salinity and drought stress. Interplay of antioxidative environment mediated by ASH, osmolytes (polyamine, proline, etc.), and strong activity of antioxidative enzyme system leads to maintenance of plastid integrity and therefore enhanced photosynthetic efficiency in colonized plant during abiotic stress. In addition, *P. indica* also induces salt and drought stress-responsive genes of the plant, which may play an important role in enhanced abiotic stress tolerance of crop plants.

24.1
Introduction

The unfavorable environmental parameters, such as drought, salinity, cold, freezing, high temperature, water logging, high light intensity, UV irradiation, nutrient imbalances, metal toxicities, nutrient deficiencies, climate change, and so on are

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termed as abiotic stress. Only 10% of the world's arable land is free of stress. Abiotic stresses have become an integral part of crop production. In general, plants suffer from dehydration or osmotic stress under drought, salinity, and low-temperature condition, which causes reduced availability of water (dehydration) for cellular function and maintenance of cellular turgor pressure. Prolonged period of dehydration leads to high production of ROS in the chloroplasts, causing irreversible photoinhibition and cellular damage. Because of cellular damage, mainly the cell membrane integrity is disturbed, and therefore plant roots are unable to absorb minerals efficiently, causing nutritional stress [1]. Plants respond to stress as individual cell and synergistically as a whole organism. Generally, stress signal is first perceived by receptors of the plant cells. Following this, the signal information is transduced, resulting in activation of various stress-responsive genes. The products of these stress genes ultimately lead to a stress tolerance response or plant adaptation and help the plant to survive and surpass unfavorable conditions [2, 3]. The response could also result in growth inhibition or cell death, which would depend on the number and type of the genes, those that are up- or downregulated in response to the stress. The overall stress response of a plant is a coordinated action of several genes encoding signaling proteins/factors, including protein modifiers (methylation, ubiquitination, glycosylation, etc.), adapters, scaffolds, and antioxidative system [2, 3]. Furthermore, plant growth-promoting fungi (PGPF) such as arbuscular mycorrhizal fungi (AMF), ectomycorrhizae, and other endophytic fungi [4], as well as plant growth-promoting rhizobacteria (PGPR) [5] or plant growth-promoting bacteria (PGPB) [6], confer abiotic tolerance and decreased yield losses in cultivated crop plants. AMF can act as a biofertilizer, bioprotectant, and biodegrader [7] and, in turn, modulate stress responses and increase the lowest limit of tolerance of the plant to abiotic stresses. Several studies are available on the impact of AMF on plant's abiotic stress tolerance, suggesting that AMF play a comprehensive role in plant's stress tolerance, and colonization of AMF induces a molecular signaling cascade that affects stomatal conductance, transpiration, photosynthesis, leaf dehydration, root hydration, hydraulic conductivity, growth, nutrient uptake, low weight metabolites (e.g., sugars, glycerol, amino acids, and sugar alcohols), and morphology. However, application of AMF in sustainable agriculture is limited due to unavailability of axenic culture and its host specificity, as AMF cannot colonize a group of important crop plants.

A recently discovered root endophytic fungus of the Sebacinaceae family, *Piriformospora indica*, can colonize the roots of many plant species, including *Arabidopsis* [8–19]. Infestation of *P. indica* can be intercellular or intracellular (Figure 24.1); however, unlike AMF, they do not form arbuscular structures in plant cells [10]. There are several reports which suggest that *P. indica* can mimic the effect of AMF colonization to plants; besides, this fungus is axenically cultivable and has a broad host spectrum. *P. indica* can provide several benefits to host plants, such as better tolerance to biotic (diseases) and abiotic stresses, for example, drought and salinity stress, and improved plant fitness by increasing growth performance under normal and stress conditions [18, 20]. The ability of *P. indica* to promote plant growth, higher seed yield, seed oil content, and so on of various host plants under conditions that are

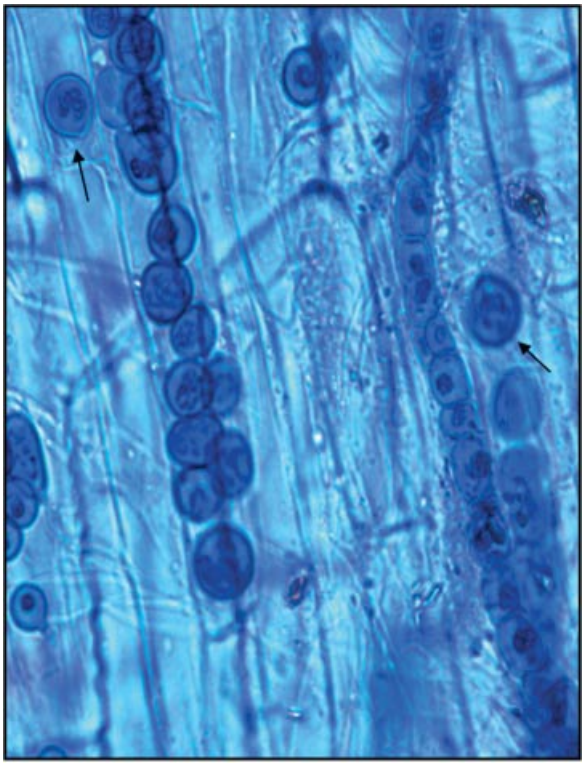

Figure 24.1 Trypan blue staining of maize plant roots showing intracellular *P. indica* chlamydospores (black arrow) observed at day 10. The fungus forms intercellular and intracellular pear-shaped chlamydospores within root. Fungus grows into cortex tissue but does not colonize vascular tissue of the root and never colonizes the shoot tissue. Adapted from Ref. [9], reprinted with permission from the Society for General Microbiology © 2009.

not optimal for the plants is well documented [8, 11, 18, 21, 22]. It can act as a bioprotector and facilitate hardening of micropropagated plants that are transferred to soil and the natural environment [23, 24]. It also has a stimulatory effect on adventitious root formation in ornamental stem cuttings. There are also studies showing that culture filtrates of the fungus are rich in nutrients and can promote growth yield [22]. However, the exact nature of plant growth promotional effects is still unclear [25, 26]. *P. indica* was reported to activate both nitrate reductase that plays a major role in nitrate acquisition and a starch-degrading enzyme, glucan-water dikinase, involved in early events of starch degradation in the plants such as tobacco and *Arabidopsis* [15]. Root infestation resulted in promotion of plant growth and yield. In this chapter, we have emphasized the effect of *P. indica* colonization on the abiotic stress tolerance and the mechanism for these stress tolerance in colonized plants. Colonization may lead to activation of various genes involved in stress acclimation and their roles in maintenance of cell homeostasis and activation of antioxidant system are also discussed.

24.2
Role of *P. indica* in Salt Tolerance

Soil salinity is a major threat to crop yield and a widespread problem. Around 7% of the global land surface is covered with saline soil [27]. Out of 1.5 billion ha of cultivated land, about 77 million ha (5%) is affected by excess salt content mainly induced by irrigation with groundwater of high salt content [28]. At present, it is a well-known fact that crop yield is low in saline soil, mainly due to decrease in efficient nutrient uptake, plant water holding capacity, and adverse effect on photosynthesis. Salinity and drought exert their influence on a cell mainly by disrupting the ionic and osmotic equilibrium [3]. Thus, excess of Na^+ ions and osmotic changes in the form of turgor pressure are the initial triggers, leading to a cascade of events that can be grouped into ionic and osmotic signaling pathways, the outcome of which is ionic and osmotic homeostasis, resulting in stress tolerance. Many crop species are extremely sensitive to soil salinity and are known as glycophytes, whereas salt-tolerant plants are known as halophytes. In general, glycophytes cannot grow at 100 mM of NaCl, whereas halophytes can grow at salinities over 250 mM of NaCl. The salinity-sensitive plants restrict the uptake of salt and strive to maintain an osmotic equilibrium by the synthesis of compatible solutes, such as amino acids and sugars. The salinity-tolerant plants have the capacity to sequester and accumulate salt in the cell vacuoles, thus preventing the buildup of salt in the cytosol and maintaining a high cytosolic K^+/Na^+ ratio in their cells. Recent studies suggested that *P. indica* could enhance the ability of plants to cope with salt stress. Waller *et al.* [18] have shown that *P. indica* colonization enhanced salt tolerance of barley plants. The detrimental effect of moderate salt stress was completely abolished by *P. indica* with a higher biomass gain. This effect of enhancement of salt tolerance by *P. indica* was similar to that of AMF. However, AMF-induced salt tolerance is due to improvement in plant nutrient uptake and ion balance, protecting enzyme activity and facilitating water uptake.

Earlier studies suggested salt-induced increase in lipid peroxidation and reduction in metabolic heat production [31] in salt-sensitive plants, while they remained unchanged in salt-tolerant cultivars. Baltruschat *et al.* [32] assessed the impact of *P. indica* colonization on biochemical markers for salt stress, such as metabolic activity, fatty acid composition, lipid peroxidation, and marked reduction in metabolic heat production in salt-sensitive barley plants. Reduction in plant metabolic activity is recognized in salt-stressed plants. Salt-induced responses, that is, heat emission and ethane production in *P. indica*-colonized salt-sensitive plant, resemble those of salinity-tolerant plants because *P. indica* increases the metabolic activity in leaves of salt-stressed plant and therefore recompenses the salt-induced inhibition of metabolic activity. Calorimetric studies indicated that the rate of metabolic activity increased in leaves of *P. indica*-infected plants after salt treatment. Prior studies have also shown that the extent of natural herbicide resistance of wild oat biotypes is tightly correlated with the rate of heat production upon herbicide exposure, owing to the activation of metabolic pathways required for defense responses [33]. This suggests that enhanced tolerance to salt stress can be associated with higher metabolic activity in *P. indica* colonized plants [32]. Salt stress can induce ionic stress and osmotic stress

in plant cell, leading to enhanced accumulation of ROS that are harmful to plant cell at high concentration. On the one hand, ROS accumulation can be toxic to living cells, causing oxidative damage to DNA, lipids, and proteins, and on the other hand, ROS can act as signaling molecules for stress responses. Several studies have demonstrated that tolerance of plants to salt stress is associated with the induction of antioxidant enzymes [34–36]. Recent reports suggest that *P. indica* induces antioxidative environment in the cell by altering the activities of different enzymes involved in ROS-scavenging system [9, 37]. Exogenously applied unsaturated fatty acids can protect barley plants during NaCl-induced stress [38]. Lipid desaturation could be an important component of plant tolerance in response to salt stress. *P. indica* colonization leads to a significant reduction in the proportion of oleic acid in barley leaves and also induces changes in fatty acid composition similar to those induced by salinity [32]. Such effects on the fatty acid composition of host plants may display a symbiotic adaptive strategy mediated by the endophyte to cope with salt stress in hostile environments [37]. Owing to its original habitat, *P. indica* might induce similar effects on fatty acid composition of the host plants [32].

It has been shown that *P. indica* is able to produce auxin when associated with plant roots [39] or changes in phytohormone synthesis and perception in plants [40–42]. Exogenous auxin has been found to transiently increase the concentration of ROS and then prevent hydrogen peroxide (H_2O_2) release in response to oxidative stress (caused by paraquat) and enhanced APX activity, while decreasing CAT activity [43, 44]. In a recent study, it was found that *P. indica* increases the level of osmolytes such as polyamines and amino acid proline (unpublished data) in colonized plants. This increase in polyamine content is due to the upregulation of methionine synthase in colonized plant, which plays a crucial role in biosynthesis of polyamines and ethylene [10]. A well-known adaptive response of plants under salt stress is the synthesis and accumulation of low molecular weight organic compounds in the cytosol and organelles, which are collectively known as compatible osmolytes because they accumulate and function without perturbing intracellular biochemistry. Transgenic tobacco plants overproducing polyamines also have enhanced tolerance toward salt stress, and salt treatment induces antioxidant enzymes such as APX, SOD, and glutathione *S*-transferase (GST) more significantly in these transgenic plants than in wild-type controls [45]. The major function of osmolytes is osmotic adjustment to counteract higher inorganic salt in vacuole and root medium, protection of membrane, and stabilization of proteins. The osmolyte proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes. Proline may also function as a protein-compatible hydrotrope and as a hydroxyl radical ($OH^{\bullet}$) scavenger [46].

In a previous study, Waller *et al*. [18] has reported that *P. indica* enhances the level of antioxidant buffer ascorbate and induces dehydroascorbate reductase activity in colonized plant. Ascorbic acid is directly involved in detoxification of H_2O_2 by coupling with glutathione cycle or NADH. Moreover, it acts directly to neutralize oxygen free radicals [47]. During early stage of salt exposure, *P. indica* maintains the redox balance ascorbate and increases its concentration in colo-

nized plant; however, over time, the concentration decreases in both the salt-treated colonized and the control plants. Several studies suggested that the tolerance of plant to salt stress is associated with the capability of detoxification of ROS, which is directly related to the induction of antioxidant enzymes [32]. Overexpression of CAT, APX, or DHAR in transgenic plants enhanced tolerance to salt stress [48, 49]. However, *Arabidopsis* double-mutant plants deficient in cytosolic and thylakoid APX also show enhanced tolerance to salinity, suggesting that ROS such as H_2O_2 could be responsible for activation of an abiotic stress signal that leads to enhanced stress tolerance [50]. Exposure to NaCl increases the activities of antioxidant enzymes CAT, APX, DHAR, MDHAR, and GR, but this initial induction of activity cannot sustain and decreases over time. However, in the presence of *P. indica*, the decrease in the enzyme activity is less pronounced and delayed. The elevated levels of GR, MDHAR, and DHAR activities affect the ascorbate level during salt exposure in *P. indica* colonized plant. Ratio of ascorbate to DHA decreased in the salt-sensitive *Lycopersicon esculentum* under salt stress and increased in the salt-tolerant *L. pennellii* [51]. Earlier, investigations have shown that ascorbate content decreased in salt-sensitive and salt-tolerant pea cultivars as well, but the decline was greater in the salt-sensitive plants [34]. The importance of ascorbate in cellular protection under salt stress has also been demonstrated in an ascorbate-deficient *Arabidopsis* mutant. Impaired in the ascorbate-glutathione cycle, this mutant accumulated high amounts of ROS and showed increased sensitivity to salt stress [52]. Constant exogenous application of ascorbate increased resistance to salt stress and attenuated the salt-induced oxidative burst [53]. Alternatively, ascorbate can improve the tolerance of barley to high salinity via processes related to root growth. Ascorbic acid and high ratio of reduced to oxidized ascorbate accelerate root elongation and increase root biomass [54].

Under salt stress condition, MDHAR activity remained elevated in roots of both salt-sensitive and -tolerant plants. *P. indica* maintains a sustainably higher CAT and APX activity in salt-sensitive plants during salt exposure. CAT activation is a well-known marker of oxidative stress and catalyzes the degradation of hydrogen peroxide into water and thus reduces the oxidative damage to cell. APX is an integral component of glutathione-ascorbate cycle and it detoxifies peroxides such as hydrogen peroxide into water using the ascorbate as substrate. Furthermore, it was found that during the interaction with *P. indica*, enhanced glutathione pool was observed in the plant leaves [18]. As glutathione is a key component in the glutathione-ascorbate cycle, it can suppress the effect of ROS on leaves and, consequently, on photosynthesis. The exact mechanism responsible for *P. indica*-mediated upregulation of the plant antioxidant system is not yet known. The hormonal signaling in the enhancement of salt tolerance of the plant in *P. indica* colonized plant cannot be rule out. *Sebacina vermifera*, an endophyte closely related to *P. indica*, downregulates ethylene production in *Nicotiana attenuata* [40]. During the interaction, induction of methionin synthase takes place, and thus an increased level of ethylene has been observed in plant root. Ethylene signaling may be required for the plant salt tolerance and it may induce some antioxidant enzyme during heat stress. However, the function of phytohormones in salt tolerance has not been clear yet.

24.3 Role of *P. indica* in Drought Tolerance

Water deficit stress is known as drought stress, which reduces agricultural production mainly by disrupting the osmotic equilibrium and membrane structure of the cell. Climate models have indicated that drought stress will become more frequent because of the long-term effects of global warming, which highlights the urgent need to develop adaptive agricultural strategies for a changing environment. Actually, the water stress within the lipid bilayer results in displacement of membrane proteins, which contributes to loss of membrane integrity, selectivity, disruption of cellular compartmentalization, and loss of membrane-based enzyme activity. The high concentration of cellular electrolytes due to the dehydration of the protoplasm may also cause disruption of the cellular metabolism. To avoid drought stress, plants close their stomata, repress cell growth and photosynthesis, activate respiration, reduce leaf expansion, and start shedding older leaves to reduce the transpiration area [1]. The components of drought and salt stress crosstalk as both these stresses ultimately result in dehydration of the cell and osmotic imbalance. Overall, drought stress signaling encompasses three important parameters [55]: (a) reinstating the osmotic and the ionic equilibrium of the cell to maintain cellular homeostasis under the condition of stress, (b) control and repair of stress damage by detoxification, and (c) signaling to coordinate cell division to meet the requirements of the plant under stress.

Recent works suggest that *P. indica* is involved not only in salt stress tolerance but also in drought stress tolerance. Sherameti *et al.* [56] has shown that *P. indica* enhances drought tolerance of *Arabidopsis*. Furthermore, the authors found that after exposure for 84 h to drought stress at the seedling stage, none of the uncolonized plant recovered and survived, while about 50% of the *P. indica*-colonized plant produced seeds. *P. indica* is also found to be involved in enhancing the drought stress tolerance of other plants, such as maize, mustard, cabbages, cress, and tobacco. The primary visible effect of *P. indica*-induced drought tolerance on the plant is shoot growth. In another study on Chinese cabbage, no visible effect of drought was seen [57]. Like salt stress, drought stress also induces strong oxidative stress and generates ROS in plant. ROS act upon the polyunsaturated lipids of membrane and thereby form malondialdehyde (MDA). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress. Drought stress promotes MDA accumulation in the leaves, while *P. indica* colonized plant contains a lower amount of MDA, suggesting that *P. indica* prevents the colonized plant from oxidative stress. Furthermore, *P. indica* induces the antioxidant enzyme activity in plant leaf during the drought stress. Three enzymes, SOD, CAT, and POD, were found induced in the colonized plant. The induction of CAT is somewhat different; it is found induced in both control and colonized plants, but the induction is higher in colonized plant. PODs are a large family of enzymes that detoxify hydrogen peroxide, organic hydroperoxides, or lipid peroxides to generate alcohols. PODs contain a heme cofactor in their active sites that is synthesized in the plastid. In addition, PODs contain redox-active cysteine residues that directly

measure the redox potential in cell or organelle. The most important organelle in the leaf that controls the redox potential in the cell is plastid. These enzymes can play an important role in the detoxification of ROS. Hence, one can understand why the amount of MDA is reduced in colonized plant.

P. indica induces not only the antioxidative enzymes but also the antioxidative molecules such as ascorbate under the drought stress. The fungus induces the accumulation of ascorbate in root and shoot, especially leaves of the plant, and maintains a higher antioxidative environment in plant cell. At the molecular level, fungus induces monodehydroascorbate reductase 2 (MDHAR2) and dehydroascorbate reductase 5 (DHAR5) in the colonized plant [58]. MDHAR2 and DHAR5 are the important part of the ascorbate–glutathione cycle that maintains ascorbate in its reduced state. MDHAR2 converts MDHA into the ascorbate using NADH, while DHAR5 converts DHA into ascorbate using its integral part, the glutathione cycle, and finally gives an antioxidative environment to the cell mediated by ascorbate. The importance of these two genes was analyzed using the knockdown (KO) lines of *Arabidopsis*, and it was found that growth, flower development, and seed production were not promoted by fungus and were inhibited under drought stress. This indicates that both the enzymes are crucial for the plant to respond to the fungus and the fungus-mediated growth promotion and cannot be fully replaced by other members of the gene family [58]. It has been demonstrated that loss of benefits for the plants could be caused by a shift from mutualism to parasitism, a phenomenon that occurred due to an uncontrolled growth of fungal hyphae in the roots [59]. During the drought stress, KO lines were found over colonized root, indicating that these mutants were less protected against the fungal colonization. Furthermore, an antifungal protein PDF1.2, which is not expressed in uncolonized root, but expressed at a detectable level in colonized root, is several times upregulated in KO lines, where interaction shifts from mutualism to parasitism. This suggests that these two genes of ascorbate-glutathione cycle contribute to the repression of defense gene expression against *P. indica* under drought stress condition [58]. Taking all the studies into consideration, activation of antioxidative system in leaves is a major target of the fungus and plastids are the main targets of drought stress in leaves.

The most crucial adverse effect of drought stress is the reduction in photosynthetic efficiency, pigment content, and proteins of the photosynthetic machinery and the biosynthetic pathways in the stroma of the plastids. *P. indica* does not target specific photosynthesis genes or proteins to establish drought tolerance, but creates preventive atmosphere in the cells that stabilizes plastid function by inducing different antioxidative enzymes. However, chloroplast is a major organelle site for antioxidative activity in the cells where SOD, APX, PODs, MDHAR, and DHAR are localized [60], and induction of these enzymes in the leaves may stabilize the plastid structure and detoxify the ROS during the drought-induced oxidative stress. The ultimate/crucial effect of *P. indica* colonization on leaves under drought condition is the increase in chlorophyll content. Drought stress also has a strong effect on photosynthesis. The photosynthetic efficiency ($F_{\text{variable}}/F_{\text{maximum}}$, F_v/F_m) values around 0.83 reflected the potential fluorescence quantum efficiency of photosystem II, which are sensitive indicators of plant photosynthesis performance [61], and the

values lower than 0.83 indicated the exposure of stress [62]. Drought stress decreases the F_v/F_m values in uncolonized plant, but colonization of fungus compensates the loss in F_v/F_m values and is equal to that of unstressed plant. The difference in the F_v/F_m values clearly demonstrates the beneficial effect of the fungus on photosynthetic efficiency under drought stress [56, 57]. Besides, *P. indica* impeded the drought-induced decline in photosynthetic efficiency and degradation of chlorophylls and thylakoid proteins [57]. Interestingly, *P. indica* does not influence any specific photosynthetic genes or protein that may lead to an increase in photosynthetic efficiency, but a plastid-localized CAS protein appears to be a specific target in the chloroplast during the drought stress. CAS protein is identified as a chloroplast-localized Ca^{2+}-sensing receptor protein that is crucial for proper stomatal regulation in response to elevations of external Ca^{2+}. CAS fulfils this role through modulation of the cytoplasmic Ca^{2+} concentration under stress condition and is also involved in signaling [63]. Drought stress induces the mRNA and protein level in the leaves and it is likely that the fungus counteracts drought stress by elevating cytoplasmic calcium transients, which finally results in stomata closure in the guard cells. A large number of genes involved in drought tolerance are more quickly and strongly upregulated in *P. indica*-colonized *Arabidopsis* leaves upon exposure to drought stress. This result was further validated when a *P. indica*-insensitive *Arabidopsis* mutant (*pii*) was found less tolerant to drought stress and did not upregulate the stress-related genes in the presence of *P. indica*. Hence, *P. indica*-mediated drought tolerance to *Arabidopsis* is associated with the priming of the expression of rather diverse set of stress-related genes in the leaves [42, 56].

These stress-related genes are involved in rather different cellular processes. Phospholipase Dδ (PLDδ) is involved in phospholipid metabolism at the plasma membrane; calcineurin B-like protein 1 (CBL1)/CBL-interacting protein kinase 3 (CIPK3) is involved in cytoplasmic signaling; histone acetyltransferase (HAT), dehydration response element binding protein 2A (DREB2A), and ANAC072 control the gene expression in the nucleus; and other proteins such as salt and drought ring finger 1 (SDIR1) and early response to dehydration 1 (ERD1) have a role in protein degradation and response to dehydration 29A (RD29A) with cytoplasmic function. RD29A and ERD1 are the reporters of drought stress responses. ERD1 is a plastid-localized caseinolytic protease that is induced by dehydration or cold stress. PLDδ is associated with the plasma membrane and specifically induced under drought stress. This plasma membrane-bound PLDδ is activated in response to hydrogen peroxide and the resulting phosphatidic acid (PA) functions to decrease hydrogen peroxide-promoted programmed cell death [64]. DREB2A is a transcription factor that specifically interacts with *cis*-acting, dehydration-responsive elements involved in drought stress-responsive gene expression in *Arabidopsis*. Intact DREB2A expression does not activate downstream genes under normal growth condition, which suggests that this transcription factor requires posttranslational modification for activation. However, the activation mechanism has not yet been clarified, but a constitutive active form of DREB2A showed improved drought stress tolerance in *Arabidopsis* [65]. SDIR1 is an E3 ubiquitin ligase localized in intracellular membranes of all tissues of *Arabidopsis* and is a positive regulator of ABA signaling, induced by drought and salt

stress but not by ABA. Overexpression of SDIR1 is involved in an ABA-dependent pathway leading to stress tolerance [66].

Calcium (Ca^{2+}) signaling is an important part of the early signaling system in plant response to various stimuli [67, 68]. Alterations occurred in cytosolic-free Ca^{2+} ($[Ca^{2+}]$cyt) in response to abiotic signals [69], such as drought [70]. Ca^{2+} acts as a secondary messenger in plant cells and links different input signals to many diverse and specific responses [68]. The Ca^{2+} signaling system is based on multifactorial processes that start with a specific Ca^{2+} signature and the availability of a specific set of Ca^{2+} sensors and end with target genes and proteins that activate precise downstream events [69, 71]. The source of the Ca^{2+} contributing to the rise in $[Ca^{2+}]$cyt (apoplasts or internal stores or both) is crucial for the physiological responses [70, 72]. Sun *et al.* [57] found that the expression levels of the drought-related genes DREB2A, CBL1, ANAC072, and RD29A were upregulated in the drought-stressed leaves of *P. indica* colonized plants. Furthermore, the CAS mRNA level of the thylakoid membrane-associated Ca^{2+}-sensing regulator and the amount of the CAS protein were also found to be increased. Thus, Ca^{2+} signaling is involved in *P. indica*-induced drought stress tolerance in plants. CBL1 is an important player in Ca^{2+} signaling and integrates plant responses to abiotic stresses, including drought stress ABA-independent pathways. Besides CBL1, a calcium sensor-associated kinase, CIPK3, has multiple functions in stress responses and might be a crosstalk node in stress and ABA signaling pathway [73]. Interestingly, CIPK3 primarily modulates cold- and salt-induced gene expression but not drought-induced gene expression; however, the level was earlier upregulated in *P. indica* colonized seedlings during the drought stress. Another transcription factor DREB1B, involved in dehydration and cold responses, works on the recruitment by HAT. Thus, *P. indica* might control gene expression more generally by regulating crucial factors involved in histone acetylation. The earlier upregulation of the genes involved in stress responses in *P. indica*-colonized plants is the modulation of the defense system by molecular signaling, which finally resulted in the prevention of water loss, balanced shift of metabolism, and functional and structural integration of the organelles and cell.

24.4 Conclusions

P. indica is a root endophytic fungus that has a broad host spectrum, including the monocot, dicot, and Brassicaceae family, which are not colonized by the mycorrhizal fungus. This interaction provides a critical linkage between the plant root and the soil. As a result, *P. indica* colonized plants are often more competitive and better able to tolerate environmental stress than the uncolonized plants. Plant responses to colonization by *P. indica* can range from growth promotion to multistress tolerance. Growth promotion in plant is the characteristic effect of the fungal colonization and visible under stress condition. This growth promotion may be due to the nutritional transfer by the fungus to the plant and phytohormone signaling mediated by auxin and cytokinine secreted by colonized fungus. On the basis of the reports available, we

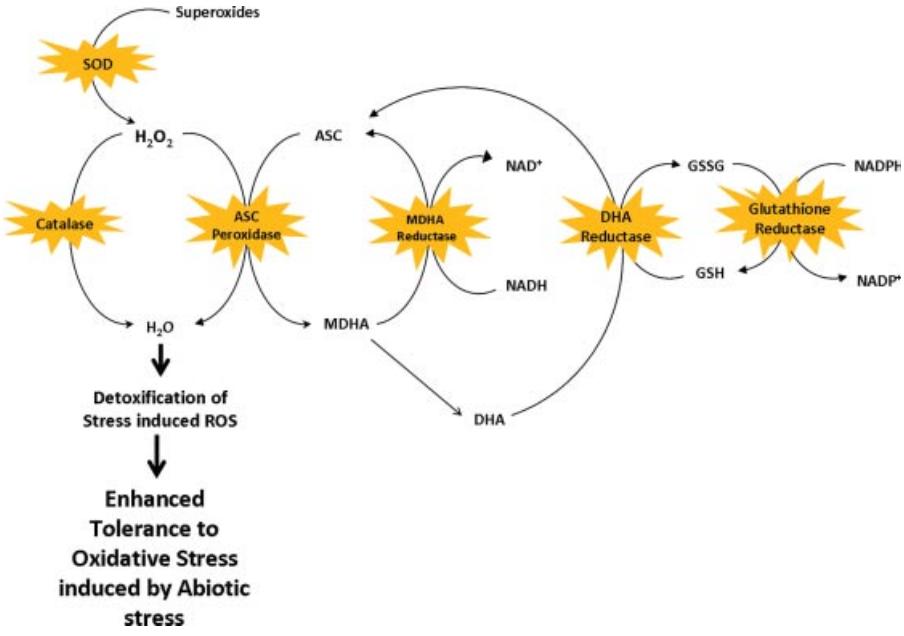

Figure 24.2 Couples series of redox reaction involved with scavenging of H2O2 of glutathione–ascorbate cycle in the plant cell. *P. indica* influences nzymes' expression and activity of the cycle (shown in exploding star) and leads to induction in ROS scavenging capacity in the cell. SOD (superoxide dismutase), ASC peroxidase (ascorbate peroxidase), MDHA reductase (monodehydroascorbate reductase), and DHA reductase (didehydroascorbate reductase) are the important enzymes playing crucial role in detoxification of stress-induced ROS and are differentially induced in *P. indica*-colonized plant during the abiotic stress.

proposed a diagrammatic representation of ROS-scavenging system of plant (Figure 24.2), showing how *P. indica* influences the glutathione–ascorbate cycle that resulted in enhanced oxidative stress tolerance under the abiotic stresses. *P. indica* modulates the defense system and alters the metabolism to compensate the loss in photosynthesis and prevention of oxidative damage due to stress. As the role of *P. indica* in abiotic stress tolerance has been shown under the controlled green house conditions, therefore we suggest the use of *P. indica* can also be tested in the agriculture field. We, further, suggest that this endophytic fungus is a good candidate for the application in sustainable agriculture to help crop plants overcome salt, drought, and other abiotic stresses.

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References

1 Tuteja, N. (2010) Cold, salinity, and drought stress, in *Plant Stress Biology: From Genomics to Systems Biology* (ed. H. Hirt), WILEY-VCH Verlag Gmbh & Co. KGaA, Weinheim, pp. 137–160.

2 Jones, H.G. and Jones, M.B. (1989) Introduction: some terminology and common mechanisms, in *Plants Under Stress* (eds H.G. Jones, T.J. Flowers, and M.B. Jones), Cambridge University Press, Cambridge, pp. 1–10.

3 Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.*, **444**, 139–158.

4 Das, A. and Varma, A. (2009) Symbiosis: the art of living, in *Symbiotic Fungi: Principles and Practice* (eds A. Varma and A.C. Kharkwal), Springer, Berlin, pp. 1–28.

5 Kloepper, J.W. and Schroth, M.N. (1978) Plant growth-promoting rhizobacteria on radishes, in *Proceedings of the 4th International Conference on Plant Pathogenic Bacteria*, vol. **II**, Station de Pathologie Vegetale et Phyto-Bacteriologie ed, Gilbert-Clarey, Tours, pp. 879–882.

6 Bashan, Y. and Holguin, G. (1998) Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. *Soil Biol. Biochem.*, **30**, 1225–1228.

7 Xavier, L.J.C. and Boyetchko, S.M. (2004) Arbuscular mycorrhizal fungi in plant disease control, in *Fungal Biotechnology in Agricultural, Food, and Environmental Applications* (ed. D.K. Arora), Marcel Dekker, New York, pp. 183–194.

8 Varma, A., Verma, S., Sudha, N., Sahay, S., Butehorn, B., and Franken, P. (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. *Appl. Environ. Microbiol.*, **65**, 2741–2744.

9 Kumar, M., Yadav, V., Tuteja, N., and Johri, A.K. (2009) Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. *Microbiology*, **155**, 780–790.

10 Peskan-Berghofer, T., Shahollari, B., Giang, P.H., Hehl, S., Markert, C., Blanke, V., Varma, A.K., and Oelmuller, R. (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. *Physiol. Plant.*, **122**, 465–477.

11 Pham, G.H., Singh, A.N., Malla, R., Kumari, R., Saxena, A.K., Rexer, K.H., Kost, G., Luis, P., Kaldorf, M., Buscot, F., Herrmann, S., Peskan, T., Oelmuller, R., Mittag, M., Declerck, S., Hehl, S., and Varma, A. (2004) Interaction of Piriformospora indica with diverse microorganisms and plants, in *Plant Surface Microbiology* (eds A. Varma, L. Abbott, D. Werner, and R. Hampp), Springer, Germany, pp. 237–265.

12 Sahay, N.S. and Varma, A. (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. *FEMS Microbiol. Lett.*, **181**, 297–302.

13 Shahollari, B., Varma, A., and Oelmüller, R. (2005) Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. *J. Plant Physiol.*, **162**, 945–958.

14 Shahollari, B., Bhatnagar, K., Sherameti, I., Varma, A., and Oelmüller, R. (2007) Molecular symbiotic analysis between Arabidopsis thaliana and Piriformospora indica, in *Advanced Techniques in Soil Microbiology* (eds A. Varma and R. Oelmuller), Springer, Berlin, pp. 307–318.

15 Sherameti, I., Shahollari, B., Venus, Y., Altschmied, L., Varma, A., and Oelmuller, R. (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. *J. Biol. Chem.*, **280**, 26241–26247.

16 Varma, A., Singh, A., Sahay, N.S., Sharma, J., Roy, A., Kumari, M., Rana, D., Thakran, S., Deka, D., Bharti, K., Franken, P., Hurek, T., Blechart, O., Rexer, K.H., Kost, G., Hahn, A., Hock, B., Maier, W., Walter, M., Strack, D., and Kranner, I. (2001) Piriformospora *indica:* a cultivable mycorrhiza like endosymbiotic fungus, *Mycota IX, Springer Series*, Springer, Berlin, pp. 123–150.

17 Verma, S., Varma, A., Rexer, K.H., Hassel, A., Kost, G., Sarabhay, A., Bisen, P., Buetenhorn, B., and Franken, P. (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia*, **90**, 896–903.

18 Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P., and Kogel, K.H. (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc. Natl. Acad. Sci. USA*, **102**, 13386–13391.

19 Yadav, V., Kumar, M., Deep, D.K., Kumar, H., Sharma, R., Tripathi, T., Tuteja, N., Saxena, A.K., and Johri, A.K. (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in the phosphate transport to the host plant. *J. Biol. Chem.*, **285**, 26532–26544.

20 Schäfer, P., Khatabi, B., and Kogel, K.H. (2007) Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism. *FEMS Microbiol. Lett.*, **275** (1), 1–7.

21 Dolatabadi, H.K., Goltapeh, E.M., Jaimand, K., Rohani, N., and Varma, A. (2011) Effects of *Piriformospora indica* and *Sebacina vermifera* on growth and yield of essential oil in fennel (*Foeniculum vulgare*) under greenhouse conditions. *J. Basic Microbiol.*, **51** (1), 33–39.

22 Bagde, U.S., Prasad, R., and Varma, A. (2011) Influence of culture filtrate of Piriformospora indica on growth and yield of seed oil in *Helianthus annus*. *Symbiosis*, **53**, 83–88.

23 Varma, A., Rai, M.K., and Sahay, N.S. (2000) Microbial-biotechnology: new-paradigms and role in sustainable agriculture, in *Microbial Biotechnology for Sustainable Development and Productivity* (ed. R.C. Rajak), Scientific Publishers, India, pp. 22–37.

24 Prasad, R., Bagde, U.S., Pushpangadan, P., and Varma, A. (2008) *Bacopa monniera* L: pharmacological aspects and case study involving *Piriformospora indica*. *Int. J. Integr. Biol.*, **3**, 100–110.

25 Druege, U., Baltruschat, H., and Franken, P. (2007) *Piriformospora indica* promotes adventitious root formation in cuttings. *Sci. Hortic.*, **112**, 422–426.

26 Pham, G.H., Kumari, R., Singh, A.N., Sachdev, M., Prasad, R., and Kaldorf, M. (2004a) Axenic cultures of Piriformospora indica, in *Plant Surface Microbiology* (eds A. Varma, L. Abbott, D. Werner, and R. Hampp), Springer, Germany, pp. 593–616.

27 Ruiz-Lozano, J.M., Azcon, R., and Gomez, M. (1996) Alleviation of salt stress by arbuscular mycorrhizal *Glomus* species in *Lactuca sativa* plants. *Physiol. Plant.*, **98**, 767–772.

28 Munns, R., Cramer, G.R., and Ball, M.C. (1999) Interactions between rising CO_2, soil salinity and plant growth, in *Carbon Dioxide and Environmental Stress* (eds Y. Luo and H.A. Mooney), Academic Press, London, pp. 139–167.

29 Hernández, J.A., Olmos, E., Corpas, F.J., Sevilla, F., and Del Río, L.A. (1995) Salt-induced oxidative stress in chloroplasts of pea plants. *Plant Sci.*, **105**, 151–167.

30 Yang, Y.L., Guo, J.K., Zhang, F., Zhaob, L.Q., and Zhang, L.X. (2004) NaCl induced changes of the H^+-ATPase in root plasma membrane of two wheat cultivars. *Plant Sci.*, **166**, 913–918.

31 Criddle, R.S., Hansen, L.D., Breidenbach, R.W., Ward, M.R., and

Huffaker, R.C. (1989) Effects of NaCl on metabolic heat evolution rates by barley roots. *Plant Physiol.*, **90**, 53–58.

32 Baltruschat, H., Fodor, J., Harrach, B.D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K.H., Schäfer, P., Schwarczinger, I., Zuccaro, A., and Skoczowski, A. (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytol.*, **180**, 501–510.

33 Stoklosa, A., Janeczko, A., Skoczowski, A., and Kie, J. (2006) Isothermal calorimetry as a tool for estimating resistance of wild oat (*Avena fatua* L.) to aryloxyphenoxypropionate herbicides. *Thermochimica Acta*, **441**, 203–206.

34 Hernández, J.A., Jiménez, A., Mullineaux, P.M., and Sevilla, F. (2000) Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defenses. *Plant Cell Environ.*, **23**, 853–862.

35 Bor, M., Özdemir, F., and Türkan, I. (2003) The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. *Plant Sci.*, **164**, 77–84.

36 Sekmen, A.H., Türkan, I., and Takio, S. (2007) Differential responses of antioxidative enzymes and lipid peroxidation to salt stress in salt-tolerant *Plantago maritima* and salt-sensitive *Plantago media*. *Physiol. Plant.*, **131**, 399–411.

37 Rodriguez, R.J., Henson, J., Volkenburgh, E., Hoy, M., Wright, L., Beckwith, F., Kim, Y., and Redman, R.S. (2008) Stress tolerance in plants via habitat-adapted symbiosis. *ISME J.*, **2**, 404–416.

38 Zhao, F.G. and Qin, P. (2005) Protective effects of exogenous fatty acids on root tonoplast function against salt stress in barley seedlings. *Environ. Exp. Bot.*, **53**, 215–223.

39 Sirrenberg, A., Göbel, C., Grond, S., Czempinski, N., Ratzinger, A., Karlovsky, P., *et al.* (2007) *Piriformospora indica* affects plant growth by auxin production. *Physiol. Plant.*, **131**, 581–589.

40 Barazani, O., Von Dahl, C.C., and Baldwin, I.T. (2007) *Sebacina vermifera* promotes the growth and fitness of *Nicotiana attenuata* by inhibiting ethylene signalling. *Plant Physiol.*, **144**, 1223–1232.

41 Schäfer, P., Pfiffi, S., Voll, L.M., Zajic, D., Chandler, P.M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U., and Kogel, K.H. (2009) Phytohormones in plant root–*Piriformospora indica* mutualism. *Plant Signal. Behav.*, **4** (7), 669–671.

42 Oelmüller, R., Sherameti, I., Tripathi, S., and Varma, A. (2009) Piriformospora indica, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis*, **19**, 1–19.

43 Joo, J.H., Bae, Y.S., and Lee, J.S. (2001) Role of auxin-induced reactive oxygen species in root gravitropism. *Plant Physiol.*, **126**, 1055–1060.

44 Pasternak, T.P., Ötvös, K., Domoki, M., and Fehér, A. (2007) Linked activation of cell division and oxidative stress defense in alfalfa leaf protoplast-derived cells is dependent on exogenous auxin. *Plant Growth Regul.*, **51**, 109–117.

45 Wi, S.J., Kim, W.T., and Park, K.Y. (2006) Overexpression of carnation S-adenosylmethionine decarboxylase gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants. *Plant Cell Rep.*, **25**, 1111–1121.

46 Smirnoff, N. and Cumbes, Q.J. (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry*, **1** (28), 1057–1060.

47 Foyer, C.H. and Noctor, G. (2000) Oxygen processing in photosynthesis: regulation and signalling. *New Phytol.*, **146**, 359–388.

48 Ushimaru, T., Nakagawa, T., Fujioka, Y., Daicho, K., Naito, M., Yamauchi, Y., *et al.* (2006) Transgenic *Arabidopsis* plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. *J. Plant Physiol.*, **163**, 1179–1184.

49 Nagamiya, K., Motohashi, T., Nakao, K., Prodhan, S.H., Hattori, E., Hirose, S.,*et al.* (2007) Enhancement of salt tolerance in transgenic rice expressing an *Escherichia*

coli catalase gene, *kat E. Plant Biotechnol. Rep.*, **1**, 49–55.

50 Miller, G., Suzuki, N., Rizhsky, L., Hegie, A., Koussevitzky, S., and Mittler, R. (2007) Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. *Plant Physiol.*, **144**, 1777–1785.

51 Mittova, V., Guy, M., Tal, M., and Volokita, M. (2004) Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. *J. Exp. Bot.*, **55**, 1105–1113.

52 Huang, C., He, W., Guo, J., Chang, X., Su, P., and Zhang, L. (2005) Increased sensitivity to salt stress in an ascorbate-deficient *Arabidopsis* mutant. *J. Exp. Bot.*, **56**, 3041–3049.

53 Shalata, A. and Neumann, P.M. (2001) Exogenous ascorbic acid (vitamin C) increases resistance to salt stress and reduces lipid peroxidation. *J. Exp. Bot.*, **52**, 2207–2211.

54 Córdoba-Pedregosa, M.C., Villalba, J.M., Córdoba, F., and González-Reyes, J.A. (2005) Changes in intracellular and apoplastic peroxidase activity, ascorbate redox status, and root elongation induced by enhanced ascorbate content in *Allium cepa* L. *J. Exp. Bot.*, **56**, 685–694.

55 Liu, J. and Zhu, J.K. (1998) A calcium sensor homolog required for plant salt tolerance. *Science*, **280**, 1943–1945.

56 Sherameti, I., Tripathi, S., Varma, A., and Oelmuller, R. (2008) The root-colonizing endophyte *Pirifomospora indica* confers drought tolerance in *Arabidopsis* by stimulating the expression of drought stress-related genes in leaves. *Mol. Plant Microbe Interact.*, **21**, 799–807.

57 Sun, C., Johnson, M.J., Cai, D., Sherameti, I., Oelmüllera, R., and Lou, B. (2010) *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *J. Plant Physiol.*, **167**, 1009–1017.

58 Vadassery, J., Tripathi, S., Prasad, R., Varma, A., and Oelmüller, R. (2009) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and *Arabidopsis*. *J. Plant Physiol.*, **166**, 1263–1274.

59 Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V.M., Nitz, I., Varma, A., Grundler, F.M., and Oelmuller, R. (2008) PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. *Plant J.*, **54**, 428–439.

60 Scandalios, J.G. (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Braz. J. Med. Biol. Res.*, **38**, 995–1014.

61 Björkman, O. and Demming, B. (1987) Photon yield of O_2 evolution and chlorophyll fluorescence at 77K among vascular plants of diverse origins. *Planta*, **170**, 489–504.

62 Maxwell, K. and Johnson, G.N. (2000) Chlorophyll fluorescence – a practical guide. *J. Exp. Bot.*, **51**, 659–668

63 Vainonen, J.P., Sakuragi, Y., Stael, S., Tikkanen, M., Allahverdiyeva, Y., Paakkarinen, V., Aro, E., Suorsa, M., Scheller, H.V., Vener, A.V., and Aro, E.M. (2008) Light regulation of CaS, a novel phosphoprotein in the thylakoid membrane of *Arabidopsis thaliana*. *FEBS J.*, **275**, 1767–1777.

64 Zhang, W., Wang, C., Qin, C., Wood, T., Olafsdottir, G., Welti, R., and Wang, X. (2003) The oleate-stimulated phospholipase D, PLDδ, and phosphatidic acid decrease H_2O_2-induced cell death in *Arabidopsis*. *Plant Cell*, **15**, 2285–2295.

65 Sakuma, Y., Maruyama, K., Osakabe, Y., Qin, F., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell*, **18**, 1292–1309.

66 Zhang, Y., Yang, C., Li, Y., Zhang, N., Chen, H., Zhao, Q., Gao, T., Guo, H., and Xie, Q. (2007) SDIR1 is a RING finger E3 ligase that positively regulates stress-responsive abscisic acid signaling in *Arabidopsis*. *Plant Cell*, **19**, 1912–1929.

67 Harper, J.F. and Harmon, A. (2005) Plants, symbiosis and parasites: a calcium signalling connection. *Nat. Rev.*, **6**, 555–567.

68 Vadassery, J., Ranf, S., Drzewiecki, C., Mithofer, A., Mazars, C., Scheel, D., Lee, J., and Oelmuller, R. (2009a) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. *Plant J.*, **59**, 193–206.

69 Sanders, D., Pelloux, J., Brownlee, C., and Harper, J.F. (2002) Calcium at the crossroads of signaling. *Plant Cell*, **14**, S401–S417

70 Knight, H., Trewavas, A.J., and Knight, M.R. (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J.*, **12**, 1067–1078.

71 Lecourieux, D., Ranjeva, R., and Pugin, A. (2006) Calcium in plant defence signalling pathways. *New Phytol.*, **171**, 249–269.

72 Knight, M.R., Campbell, A.K., Smith, S.M., and Trewavas, A.J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature*, **352**, 524–526.

73 Kim, K.N., Cheong, Y.H., Grant, J.J., Pandey, G.K., and Luan, S. (2003) *CIPK3*, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. *Plant Cell*, **15**, 411–423.

25
The Micromics Revolution: MicroRNA-Mediated Approaches to Develop Stress-Resistant Crops

Camilo López and Álvaro L. Pérez-Quintero

In the past two decades, small silencing RNAs (ssRNAs or sRNAs) have emerged as one of the most exciting and revolutionary discoveries in molecular biology. ssRNAs are noncoding molecules used by eukaryotes in transcriptional and posttranscriptional gene silencing, endogenous mechanisms for gene regulation and defense against invasive nucleic acids. MicroRNAs (miRNAs), a type of ssRNAs, play a pivotal role in endogenous regulation of gene expression via cleavage or translational repression of complementary "target" mRNA molecules. Plant miRNAs were first described in *Arabidopsis* in 2002 and now have been identified in more than 120 plant species (including crops and model species); thus, plant miRNA research is one of the fastest growing fields in molecular biology. The first families of miRNAs identified were known to mainly target transcription factors and were involved in growth and development regulation. The initial tools for miRNA identification were often time consuming, expensive, and difficult. In the last few years, various "omics" approaches have been developed to study miRNAs, allowing the spread of miRNA studies to a larger number of plants and the discovery of novel miRNAs and their functions. Notably, various miRNAs are now known to play a role in biotic and abiotic stress, which has led researchers to consider them as a promising tool to develop stress-resistant crops. In this chapter, we review the role miRNAs play in the way plants react to biotic and abiotic stress. We highlight the new methodologies being used to discover miRNAs and to understand their functions in plant responses to environmental stimuli. We also discuss and emphasize the possibilities of generating crop plants with increased stress tolerance using microRNA-mediated technologies.

25.1
Introduction: Silent Molecules Scream Out Loud

There is a clear reason why RNA is at the core of the central dogma of molecular biology. Although it was once regarded as a simple mediator between DNA and

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protein, we now recognize its role in various cellular processes and its importance as being a possible precursor to all life forms. In the past 20 years, a new breakthrough in the "RNA world" has significantly changed not only our perception of the RNA's role in the cell but also our general ideas on genetics and molecular biology: the small silencing RNAs (ssRNAs).

The story begins in 1990 when plants from the genus *Petunia* were genetically transformed with a chalcone synthase gene expecting to obtain deep purple flowers but getting instead completely white flowers. The phenomenon was called cosupression and could not be fully explained at that moment [1, 2]. This silencing phenomenon was then described in plants as well as in animals and fungi, receiving names such as RNA interference (RNAi), quelling, and posttranscriptional gene silencing (PTGS) [3–5].

We now know that gene silencing is present in most groups of eukaryotes and it is governed by small silencing RNAs (ssRNAs). ssRNAs are 18–30 nucleotides (nt) long molecules that interact with complementary nucleic acids and are involved in the control of gene expression, defense against invasive nucleic acids and transposons, heterochromatin regulation, and epigenetic modifications [6]. All ssRNAs share some similarities in their biogenesis and their action mechanism. They originate from exogenous or endogenous double-stranded RNAs (dsRNAs) that are processed by RNase enzymes, Drosha, Dicer, and/or Dicer-like (DCL), to their final functional size, and then interact directly with Argonaute (AGO) proteins that associate with other proteins forming the RNA-induced silencing complex (RISC). Alternatively, they can also be incorporated into the RNA-induced transcriptional silencing (RITS) complex. Both complexes will use the ssRNA as a template to recognize and modify complementary nucleic acids [7–9].

Among the different classes of ssRNAs, microRNAs (miRNAs) (~21–22 nt) and small interference RNAs (siRNAs) (21–24 nt) have been extensively studied. miRNAs and siRNAs are chemically indistinguishable and can produce silencing in a similar manner. They differ in their biogenesis: siRNAs commonly originate from invading or aberrant nucleic acids (transposons, viruses, and transgenes), whereas miRNAs originate from endogenous genes. Another important distinction is that siRNAs act in *cis*, silencing the same molecule from which they are derived, while miRNAs act in *trans*, silencing mRNAs from other genes [9–11].

Recently, various types of ssRNAs have been described. Among these, we find the *trans*-acting siRNAs (ta-siRNAs) (21–22 nt), a type of ssRNA that combines both siRNA and miRNA pathways since they originate from a nuclear *TAS* gene that is transcribed to an mRNA, which is then cleaved by a miRNA. The cleaved product is then processed into siRNAs directed against another mRNA, thus acting in *trans* [12, 13]. There also are natural antisense transcript-derived siRNAs (nat-siRNAs) (21–24 nt) that are quitar porque tambien hay trans-acting nats siRNAs derived from naturally occurring overlapping regions of sense and antisense transcripts [14]. The heterochromatic siRNAs (hcRNAs) or repeat-associated siRNAs (ra-siRNAs) (~24 nt) are derived from transposons, repeat elements, and heterochromatin regions. They function in the RNA-dependent DNA methylation pathway by mediating histone

modification and/or DNA methylation at the target sites [15, 16]. The long siRNAs (lsiRNAs) (30–40 nt) depend on DCL1 and AGO7 for their biogenesis and function by decapping or by 5′-3′ degradation of target mRNAs [17]. The long miRNAs (lmiRNAs) (24 nt) are involved in asymmetric DNA methylation [18]. Special cases found only in animals are the relatively long scan RNAs (scnRNAs) (~29 nt), which are involved in genome editing in *Tetrahymena thermophila* [19]. The piwi-RNAs (piRNAs) (24–31 nt) are coded in large clusters and are processed in a manner independent of Dicer; they associate specifically with the PIWI subfamily of AGO proteins and are restricted to metazoans [20, 21].

There has undoubtedly been a revolution in molecular biology caused by these ssRNAs; this revolution is driven not only by the implicit importance of these molecules in eukaryote molecular biology but also by their potential applications in various areas. They include prospective developments for gene therapy, cancer, stem-cell research, and antiviral treatments for humans and animals [22] as well as important improvements in various plant traits [23].

25.2
The Silence within: Plant miRNA Pathway

miRNAs were first discovered in *Caenorhabditis elegans* in the early 1990s, in which genetic screenings in a mutant with developmental abnormalities revealed a short noncoding RNA (*lin-4*) complementary to a coding gene (*lin-14*) to be responsible for the phenotype [24]. The molecular mechanism became clearer after it was determined that the phenotype was due to a gene silencing process guided by dsRNAs [3, 25, 26]. It took some time until the full picture of the miRNAs was revealed, and it was not until 2002 that miRNAs were discovered for the first time in plants [27, 28]. We now know that these miRNAs act in similar silencing pathways and are present in many eukaryotes.

In plants, miRNAs originate from nuclear genes (named *MIRNA* genes) and are transcribed by RNA polymerase II into primary miRNAs (pri-miRNAs), with sizes varying from 100 nt to a few kilobases. A DCL protein processes the pri-miRNA into a precursor miRNA (pre-miRNA: 70 to 400 nt). This pre-miRNA forms a characteristic hairpin-like structure. A subsequent processing step by DCL slices the pre-miRNA to form a miRNA:miRNA* duplex (21–22 nt). The duplex is then methylated and exported from the nucleus to the cytoplasm where it will join an AGO protein to form the silencing complex (RISC). Only the mature miRNA strand, which is usually the one with less stable 5′ end pairing, is retained in the complex, while the passenger (miRNA*) strand is degraded. This complex will guide the silencing of complementary mRNAs (targets) [10, 29, 30].

One important difference between animal and plant miRNAs is the higher degree of complementarity in the miRNA:target pair in plants to produce effective silencing. Other differences include the targeted region in the mRNA (any mRNA region for plants and 3′ UTR for animals,) and the outcome of miRNA silencing (mostly mRNA cleavage in plants and translational repression in animals) [31, 32].

Since their first discovery in *Arabidopsis*, the repertoire of known miRNAs has grown to include more than 120 miRNA families from more than 120 plant species [33]. Most of the described miRNAs are known to target transcription factors and play a role in different developmental processes such as leaf polarity and shape, vascular tissue and meristem development, transition from juvenile to mature growth phase, flowering time and flower organ identity, stomatal development, and so on [34–37]. There is also mounting evidence of their importance in biotic and abiotic stress responses in plants [38, 39].

25.3
The Next-Generation Microscope: New Tools for miRNA Studies

To date, too few miRNAs have been studied and even less discovered using traditional "forward" genetic approaches [40, 41]. Most miRNAs are coded by multiple loci in the genome and show in consequence functional redundancy. For this reason and their small size, the identification of miRNAs by loss-of-function mutant screens was often too difficult [29]. Reverse genetics is, therefore, the tool of choice for miRNA studies. To identify new miRNAs, two approaches were traditionally used, often simultaneously, computational prediction of miRNAs and/or cloning and sequencing of small RNAs. Computational approaches relied on various characteristics of known miRNAs such as their conservation among species (both primary and secondary structure), their position in intergenic regions or introns, and the high-energy folding for the miRNA precursors. These approaches were useful only in plants with sequenced genomes or with large sets of ESTs (expressed sequence tags) or GSS (Genome Survey Sequences) [42, 43].

Direct cloning of small RNA libraries does not need fully sequenced genomes, and was the method used to discover many plant miRNA families. Briefly, this method involves (i) RNA isolation and recovery of the ssRNA fraction in an acrylamide gel, (ii) ligation of adapters, (iii) reverse transcription, and (iv) cloning and sequencing by traditional methods. This was often time consuming and expensive, and some miRNAs with low expression were difficult to obtain [29, 43].

To identify stress-responsive miRNAs, it is crucial to determine differential expression of miRNAs in different conditions. Northern blot hybridization and microarray analyses were often used for this purpose, with the disadvantage of not giving an accurate quantification. Quantitative PCR (qPCR)-based strategies were then developed for quantifying miRNA and miRNA precursors and have proved to be effective [43]. Although these previous methods are being used and constantly improved upon, miRNA investigation seems to be moving in a new direction propelled by next-generation sequencing technologies.

The sequence-by-synthesis (SBS) technology or Solexa/Illumina sequencing [44] seems to be the most suitable strategy for miRNA identification and expression studies. This approach uses reversible nucleotide terminators to sequence short DNA fragments (signatures). The processing prior to sequencing involves basically the same steps as traditional sequencing except for cloning (reverse transcription

and adapter ligation). Although the sequence length of SBS is short (around 100 bases at present) compared to other technologies such as 454 or pyrosequencing (400 bases), it is long enough to capture the full-length sequence of small RNA molecules. More importantly, with SBS millions of signatures in parallel are obtained. This enhanced capacity is enough to capture almost all the small RNA molecules present in a given sample [45]. Also, Solexa/Illumina sequencing allows a more accurate quantification of miRNAs, based on the number of reads [46]. This has allowed extensive studies on miRNA expression under different conditions in plants [47–49].

After miRNA identification and expression quantification, the next logical step is the miRNA target prediction and validation. There is software available for target prediction, constructed on base-to-base complementarities, pairing energy, or both [50, 51]. Target prediction is considerably easier in plants than in animals given their higher degree of complementarity in the miRNA:target pair, although there are still some obstacles to overcome for an accurate target prediction such as target accessibility and prediction of translational repression [52, 53].

To validate miRNA targets, quantification using Northern blot was commonly used. It was usually accompanied by transformation approaches such as miRNA overexpression. A cheaper and more powerful method is based on 5′ RACE. This method detects *in vivo* products of miRNA cleavage by reverse transcription and PCR of the 5′ end of a cleaved miRNA target. The sequencing of the PCR product reveals the precise position of target cleavage [54].

Target validation, like miRNA discovery, has been revolutionized by next-generation sequencing technologies. Recently, a pair of methods has been developed to allow deep and simultaneous sequencing of all mRNAs cleaved by miRNAs at a given stage: the degradome sequencing methods. The strategy involves capturing either uncapped polyadenilated mRNAs or capped nonpolyadenilated mRNAs, which are miRNA-cleaved products that are then sequenced using next-generation technologies. These methods also have the additional advantage of being useful to identify new targets that were not predicted by bioinformatics [55–57].

Some of the disadvantages of next-generation sequencing for both miRNA discovery and target validation include their cost, the large data sets generated, and the work on bioinformatics needed to process the data. The last is especially problematic because there is not yet clear consensus on the computational methods used for the analysis of these data. However, as these technologies get cheaper and bioinformatics software becomes more sophisticated, miRNA research will be more accessible to different groups worldwide.

25.4
Small Weapons for the Arms Race: Plant miRNAs and Biotic Stress

Plants are commonly attacked by pathogenic bacteria, fungi, viruses, insect pests, and nematodes. Understanding and controlling these attacks is of special agronomical

interest given the enormous economical losses and the human welfare risks associated with plant diseases. Many advances have been gained in the understanding of plant–pathogen interaction with the aim of developing biotic stress-resistant plants. miRNAs have not been excluded from molecular plant pathology and the importance of this pathway in both plant defense and pathogen attack seems to be greater than was once thought for plants.

A general mechanism of miRNA-mediated "antipathogen" defense is represented in Figure 25.1. This mechanism involves mainly an increased expression of miRNAs targeting negative regulators (or repressors) of plant defense, as well as the repression of miRNAs targeting positive regulators of plant defense, thus activating or enhancing defense responses. In the case of viruses, direct targeting of viral genomes by plant miRNAs may also be involved. Pathogen counterdefense is exerted mainly by suppressing the silencing pathway. We will next examine this general model in detail.

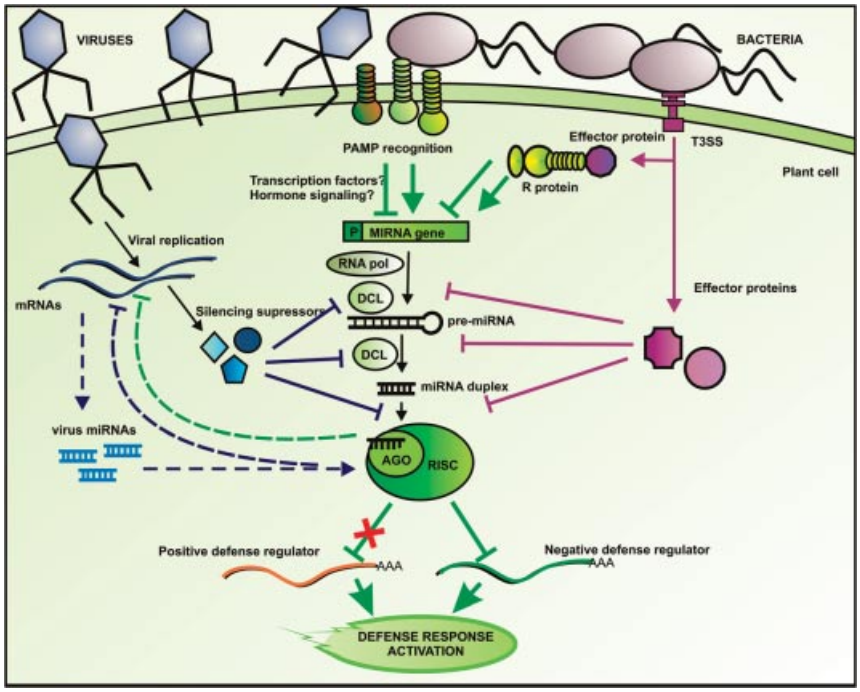

Figure 25.1 General mechanism of miRNA-mediated plant–pathogen interactions. Discontinuous lines indicate hypothetical pathways. T3SS: type-3 secretion system; PAMP: pathogen-associated molecular pattern; R protein: resistance protein; DCL: Dicer-like; AGO: Argonaute; RISC: RNA-induced silencing complex; RNA pol: RNA polymerase.

25.4.1
Suppress and Conquer: Viruses versus Plant miRNAs

The main plant defense against viruses is mediated by the siRNA pathway, through the generation of virus-derived siRNAs (vsRNAs). In this mechanism, dsRNAs are produced during viral replication and are then processed into primary and secondary siRNAs that silence mRNAs from the virus and stop its replication [39]. The siRNA pathway can be considered an "adaptive" response in the sense that virtually any replicating virus could be recognized and silenced by the plant. It is also effective because the signal is amplified and transferred from cell to cell preventing the spread of the virus to noninfected cells [58, 59].

Since their discovery, miRNAs have been postulated as important molecules in plant antiviral response. A few evidences support this hypothesis: the presence of viral suppressors affecting the miRNA pathway [60], the presence of conserved 20–24 nt sequences between plant and virus genomes [61], the differential susceptibility to virus in miRNA defective plants [62, 63], and the increasing amount of evidence of antiviral activity of miRNAs in animals [64].

Because virus nucleic acids interact directly with the host, miRNAs could be a suitable tool to affect the outcome of the virus–host interaction. Plant miRNAs could target viral genomes to directly impair their replication. This has so far been demonstrated in various cases in animal-infecting viruses [64–66]. The possibility of this process naturally occurring in plants has long been discussed, bearing in mind the possible disadvantages that the miRNA pathway has with respect to the siRNA pathway. Mainly, the miRNA pathway is not an adaptive response since the evolution of viral genomes would be fast enough to surpass the evolution of miRNAs, rendering the former ineffective in the short term [67, 68].

Employing a bioinformatics approach, we showed that several endogenous plant miRNAs have potential antiviral activity through complementarity with plant-infecting viral genomes, including miRNA families known to be differentially regulated under viral infection [65]. This is also strengthened by experiments using artificial miRNAs (amiRNAs) showing that the miRNA pathway can be effective in antiviral defense and that target complementarity is enough to impair viral replication (see Section 25.7.2) [66–68]. Our results may indicate a supporting antiviral function of the miRNA pathway to the siRNA defense mechanism or they may reflect an ancestral antiviral role for miRNAs [65].

Viruses could also encode miRNAs directed against the plant genomes (or even their own genomes) that will use the host miRNA machinery to be processed and execute their silencing effect. This mechanism has been described only in animal-infecting viruses [69, 70]. So far, the closest mechanism found in plants is encoding of sRNAs by the cauliflower mosaic virus (CaMV) that share complementarity with *Arabidopsis* genome regions [71].

There could also be indirect mechanisms mediated by miRNAs that could affect plant–virus interactions. For example, the expression of plant miRNAs targeting plant transcripts can be altered in response to virus recognition to affect the viral replication and spread. Indeed various plant miRNAs are now known to be

upregulated or downregulated after viral infection [72–77]. However, the effect of this differential regulation in the outcome of the interaction is not well established in most cases. There are some exceptions where a clearer role for miRNA regulation has been established: miR1885 is induced in response to Turnip mosaic virus infection in *Brassica rapa* and is known to target a TIR-NBS-LRR class disease-resistance gene [76]. In the case of miR164, it has been proved that its induction upon viral infection is due to hormone-dependent specific transcriptional activation [74]. Notably, next-generation sequencing or microarray methods have not being used yet for miRNA profile expression studies under viral infection, so the regulation of many miRNA-mediated processes upon viral infection is still unknown.

Nevertheless, viruses count on a powerful tool to interfere with the plant silencing machinery: the viral suppressors of gene silencing. These suppressors are proteins coded by the virus that affect siRNA and miRNA biogenesis in different ways. Some interfere with the processing of dsRNAs into ssRNAs [78], while others can interfere with the loading of sRNAs into RISC through ssRNA sequestration, can directly interfere with AGO proteins [79, 80], or may interfere with other processes including ssRNA methylation and spread of ssRNAs between cells [81]. The abundance of silencing suppressors among viruses reflects the strong selective pressure that plant sRNA-mediated silencing imposes on viral replication.

25.4.2
Being Effective: Bacteria versus Plant miRNAs

Plant resistance proteins have being considered the main molecules involved in plant defense against pathogens. An arms race model of protein evolution has been proposed for this system. In this model, a first layer of plant defense is achieved through the recognition of common features of microbes called PAMPs (pathogen-associated molecular patterns) or MAMPs (microbe-associated molecular pattern) by PAMP receptors called PRRs (pattern recognition receptors) [82]. This recognition triggers a series of events including callose deposition, ROS (reactive oxygen species) production, ion fluxes and reprogramming gene expression. This mode of action is usually known as PAMP-triggered immunity (PTI) [82]. To overcome PTI, bacteria have developed proteins called effectors that are injected into the plant cell through the type III secretion system (a syringe-like complex capable of secreting proteins) [82]. These effectors affect the PTI, thus resulting in a successful infection. In response, plants have developed resistance (R) proteins that specifically recognize bacterial effectors and trigger a specific defense response. This mechanism is usually known as effector-triggered immunity (ETI) [82].

Plant–bacteria interactions, unlike the virus case, do not involve nucleic acid exchange as a general mode of action (with known exceptions as with the genus *Agrobacterium*). Therefore, the antibacterial function of miRNAs may be exerted fundamentally through the regulation of host gene expression. Such mechanism was first revealed in the interaction between *Arabidopsis* and *Pseudomonas syringae* pv. tomato strain DC3000 (PtoDC3000), where miR393 was found to be induced in response to the flagellin-derived peptide flg22, the most-studied PAMP. This miRNA

targets the F-box family of auxin receptors TIR1 [83]. TIR1 silencing impairs auxin signaling, consequently restricting bacterial growth. These results did not only demonstrate the role miRNAs play in antibacterial defense but also implied a previously unknown importance of auxin in pathogen defense [83]. It was later discovered that PtoDC3000 can use effectors to suppress the miRNA pathway to promote disease. Effector proteins, AvrPto and HOPT-1, were found to interfere with miRNA accumulation through DCL1 and AGO activity, respectively [84]. Taken together, these results suggest an important role plant miRNAs play in response to bacteria.

Some other miRNAs have been identified to be differentially expressed under bacterial infection. Notably, miR167 and miR160 are both induced in response to PtoDC3000 and target auxin responsive factors [39, 48]. However, it has also been found in *Arabidopsis* that miR160 and miR393 expression is reduced in response to infection by *Agrobacterium tumefasciens*, with a consequent increase in the auxin level [85]. This suggests that some miRNAs could act in a pathogen-specific manner upon bacterial infection.

miRNAs 162, 168, and 825 have been shown to be downregulated in response to PtoDC3000 [48]. miR162 and miR168 regulate elements of the miRNA pathway (DCL1 and AGO1). The repression of these miRNAs would produce an overall increase in miRNA silencing activity, thus revealing a bacteria-responsive regulatory loop in the miRNA pathway and a crucial role for DCL1 and AGO in antibacterial defense [86, 87]. Recently, it has been confirmed that AGO1 plays a crucial role in PTI because AGO-impaired plants are more susceptible to bacterial infection and show decreased callose deposition and gene expression in response to PAMPs [49]. miR825 targets Cu/Zn-superoxide dismutases (SOD) and may play a role in ETI since its expression was detected to be downregulated specifically upon recognition of avrRpm1 and avrRpt2, two Pseudomonas effectors [88].

The repertory of PAMP-responsive miRNAs has been recently expanded in the *Arabidopsis*-PtoDC3000 system through the next-generation sequencing analysis, which allowed the identification of 16 upregulated miRNAs, among them not only the already known miR167 and miR160 but also miR158, miR169, miR391, and miR396 [49]. Eleven downregulated miRNAs (including miR156, miR398, and miR773) were also reported [49]. However, the mechanism by which bacteria-responsive miRNAs undergo transcriptional regulation has not been extensively studied. The promoter region of miR393 seems to be PAMP responsive, but the signaling between PAMP recognition and miRNA induction is unknown [89].

Studies on bacteria-responsive miRNAs outside *Arabidopsis* have not been published. However, our preliminary study, employing next-generation sequencing, allowed us to identify 14 upregulated and 10 downregulated conserved miRNA families during infection by *Xanthomonas axonopodis* pv. manihotis in *Manihot esculenta* (cassava), an important food crop in Third World countries. These include some known bacteria-responsive miRNAs such as miR160, miR167, and also new ones such as miR394, miR477 (both upregulated), and miR535 (downregulated). We have also identified bacteria-responsive miRNAs that are not shared with *Arabidopsis* such as miR2911 (upregulated, and probably restricted to the close relatives of cassava and *Populus*) (Pérez-Quintero *et al.*, unpublished results).

Other types of ssRNAs have been identified to mediate antibacterial responses, including a nat-siRNA induced upon R protein recognition of effector avrRpt2 in *Arabidopsis*; this nat-siRNA targets a negative regulator of ETI [90]. A lsiRNA is also responsive to PtoDC3000 infection and silences another negative regulator of ETI [17]. Both these ssRNAs make the plants resistant to bacterial infection.

In addition, miRNAs also play a role in regulating the interaction with beneficial bacteria. For example, in *Medicago truncatula* miR169 regulates the expression of transcription factor MtHAP2-1, involved in nodule development in response to *Rhizobium* infection. The expression of miR169 restricts the action of MtHAP2-1 to the nodule meristematic zone, thus promoting nodule differentiation [91]. Recently, various studies have addressed the role of miRNAs in symbiotic interactions with nitrogen-fixing bacteria, specifically in *M. truncatula* and soybean (*Glycine max*). Different miRNAs appear to be involved in various stages of bacteria colonization and root nodule formation. These include some of the miRNAs involved in the regulation of the auxin-silencing pathway such as miR160 and miR393 [92]. Other families known to be involved in symbiotic interactions are miR168 and miR159, which are differentially expressed during nitrogen-fixing bacteria recognition [92], and miR172, highly expressed in mature nodules [93]. Some species-specific miRNAs such as miR222, miR283, and miR235 in soybean may also play a role in symbiotic interaction [93]. Notably, most miRNAs known to be involved in interaction with beneficial bacteria are conserved among a wide group of plants including plants that do not form nodules.

25.4.3
To be Determined: Other Plant Pathogens and miRNAs

Too little is known about the role of miRNAs in plant interaction with other pathogenic organisms such as oomycetes, fungi, nematodes, and insects. With the notable exception of miRNAs role in the interaction of *Pinus taeda* with the rust fungus *Cronartium quercuum* f. sp. *fusiforme*, where 10 families are known to be downregulated in galls induced by the pathogen, including various species-specific miRNAs that target resistance protein, [94]. Undoubtedly, this is an exciting field to be explored and hopefully the new technologies will serve to enrich our knowledge of miRNAs role in interaction with various pathogens.

25.5
A Versatile Response to a Changing Environment: miRNAs and Abiotic Stress

As sessile organisms, plants cannot move to escape from extreme and changing conditions of the environment. Thus, plants have evolved different strategies to respond to these changes: morphological adaptations, physiological responses, and, at the molecular level, a fine-tuned reprogramming of gene expression. Regulating transcription initiation has been classically considered the main mechanism to

control gene expression. In consequence, several transcription factors involved in response to abiotic stresses have been discovered and characterized. With the discovery of miRNAs, a new layer of complexity in regulation of gene expression has been added. Several miRNAs have been recently identified to be responsive to different abiotic stresses; some of these are represented in Figure 25.2. This section describes the role the miRNA pathway plays in response to abiotic stress.

25.5.1
Elementary: Soil Elements' Uptake and miRNAs

Macro- and micronutrients in soils are essential to plants; however, the availability of these nutrients is not homogeneous in all soil types. Plants have developed different strategies to respond to the fluctuating nutrient concentrations in the environment including miRNA regulation [95]. One of the most studied miRNAs associated with abiotic stress is miR399, which is involved in the response to phosphorus (P) stress conditions. First identified in *Arabidopsis* and rice [96], miR399 is induced under low P conditions and targets a gene encoding for an ubiquitin-conjugating enzyme

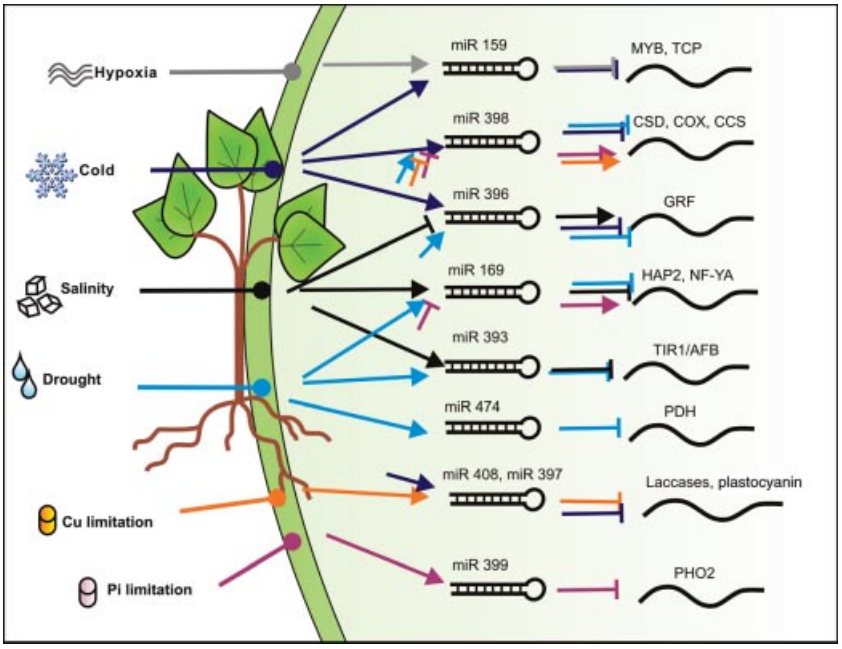

Figure 25.2 Some miRNA families involved in abiotic stress responses and their targets. Arrows indicate induction and blocked lines indicate inhibition. Some arrows have been shortened and some relations have been obviated for a better understanding of the figure. MYB and TCP: transcription factor families; CDS: Cu/Zn-superoxide dismutases; COX: cytochrome-*c* oxidase; CCS1: chaperone; GRF: growth regulation factor; HAP2 = NF-YA: nuclear transcription factor Y alpha; TIR1: transport inhibitor response 1; ARB: auxin F-box (AFB); PDH: proline dehydrogenase; PHO2: phosphate 2, ubiquitin-conjugating enzyme.

E2 [96], named *PHO2*, which is subsequently downregulated as miR399 is induced [97–100]. When expressed, the miRNA (miR399) and its target (*PHO2*) colocalize in the vascular cylinder of the root [98]. *Arabidopsis* transgenic plants that overexpress miR399 do not show accumulation of *PHO2* even under high P concentration, accumulate more P in shoots than wild plants, and show classical P toxicity symptoms (chlorosis and necrosis) [99]. The fact that toxicity observed in transgenic plants overexpressing miR399 was caused by the enhanced Pi uptake and the retention of P in old leaves suggests a transmission of a shoot-derived signal to roots that should move through the phloem [98, 100]. Recently, it was demonstrated that this signal is, in fact, the miR399 itself, which moves from shoots to roots conserving their function in these distal cells [101, 102]. miR399 seems to specifically function in response to P deficiency since it is not regulated in response to other nutrients (nitrogen, potassium, sulfate, or carbohydrates) [97, 99]. In addition, *PHO2* is protected from miRNA-mediated degradation to reduce the content of P in the shoots; this is achieved through the expression of the noncoding endogenous gene *IPS1* (induced by phosphate starvation 1), whose sequence is complementary to miR399, but it is not cleaved by it and is therefore employed to sequester the miRNA and allow *PHO2* accumulation [103]. This mechanism is known as target mimicry and has great potential for genetic transformation (see Section 25.7.1).

The use of large-scale genomics approaches allowed the identification of additional induced (miR156, miR778, miR827, and miR2111) and repressed (miR169, miR395, and miR398) miRNAs in *Arabidopsis* under phosphate deficiency [102, 104]. In soybean, using miRNA-based microarrays made it possible to identify 57 P-responsive miRNAs from 27 families. Various *cis*-elements related to phosphorus response were also found such as PHT1 element (high-affinity phosphate transporter 1) and PHR1 element (regulator of P-deficiency response) [105].

Regarding other nutrients, to study miRNA responses to nitrogen (N), the gene expression profile from specific root cell types was measured in response to an N influx. On the basis of the gene expression data, it was demonstrated that miR167 was repressed in response to nitrogen and concomitantly the expression of its target *ARF8* (an auxin response factor, ARF) was induced. Transgenic plants overexpressing miR167 showed loss of nitrogen control over lateral root emergence [106]. Other N-responsive miRNAs include miR169 and miR398 [102].

In the case of iron, some miRNAs are known to be induced under iron deprivation conditions in *Arabidopsis* including miR159a, miR169b, miR169c, miR172c, miR172d, miR173, miR394a, and miR394b [107]. An *IDE* (iron deficiency-responsive element) was found in the promoter region of some of these miRNAs [107].

On the other hand, the soil may contain not only nutrients but also potentially harmful contaminants such as heavy metals. Plants growing under conditions simulating heavy metal stress (excess or limitation) have displayed differential expression of some miRNA families. Three miRNA families are induced under low concentrations of copper (Cu) in *Arabidopsis*: miR397, miR408, and miR857. These miRNAs target laccases and plastocyanin, two secreted copper-containing proteins [108]. In the case of the miR397:laccases pair, expression of target proteins increases as copper concentration increases and is negatively correlated with miRNA

expression. However, proteins with similar function, but not regulated by the known miRNAs, display similar behavior (perhaps, regulated by unknown miRNAs) [108].

In *M. truncatula*, 38 stress-responsive miRNAs were identified in plants exposed to high concentrations of cadmium (Cd), including the induced miR171, miR319, miR393, and miR529 and the repressed miR166 and miR398; of this set, miR393 and miR171 (both induced) were experimentally validated [109]. Curiously, these families were repressed in *Brassica napus* under similar Cd conditions [110]. In rice, using next-generation sequencing of small RNAs from plants exposed to toxic concentrations of Cd, it was possible to identify several miRNAs with tissue-specific differential expression, including miRNAs induced mainly in roots (miR601, miR602, and miR603) and miRNAs downregulated in leaves and roots (miR602 and miR606 in leaves and miR604 in roots) [111]. Among these, miR604 targets a gene coding for a lipid transfer protein (LTP) previously associated with response to external stimuli [111].

25.5.2
The Silent Treatment: Cold and miRNAs

In response to low temperature, plants switch on a series of responses to overcome this condition, given the potential risks cold represents to the overall physiology of the plant. Initially, mir393 and miR319 were found to be induced and miR298 to be downregulated by cold in *Arabidopsis*; notably these miRNAs were also responsive to salinity, dehydration, and ABA [96]. Recently, through microarray analyses miR396, miR397, miR172, miR169, miR408, miR168, miR171, miR393, miR319, miR165, and miR400 were added to the list of differentially expressed miRNAs under cold conditions in *Arabidopsis* [112]. This miRNA data was subsequently employed to develop a computational algorithm to predict and annotate additional miRNAs that were further validated experimentally [113], managing to include miR156/157, miR164, miR394, and miR398, as well as cold-responsive miRNAs [113].

Brachypodium distachyon shows cold tolerance and constitutes an excellent model to understand the role of miRNAs in low-temperature response. Recently, several miRNAs were identified to be expressed in *B. distachyon* plants grown under cold conditions, including 27 conserved families and, more interestingly, 129 nonconserved [114]. Three known miRNAs (miR169, miR172, and miR397) were induced by cold stress, and novel miRNAs miR911, miR912, miR913, miR917, and miR918 showed clear changes in their expression under cold treatment [114]. In *P. thricocarpa*, another set of 19 cold stress-responsive miRNAs was identified from microarray data, 15 induced and 4 repressed [115].

In general terms, the targets predicted by these cold stress-responsive miRNAs include proteins with a broad spectrum of cellular functions, indicating a complex regulation network in responses to low-temperature conditions. Promoter analysis for some of these miRNAs has allowed the identification of several low-temperature-responsive elements (LTRs) [112] as well as other stress-responsive motifs such as the ABA response elements (ABRE), MYB binding sites, and heat stress-responsive elements (HSE) [113].

25.5.3
Out of Breath: Hypoxia and miRNAs

Hypoxia or low availability of oxygen is a known cause of stress in plants. One of the most typical conditions favoring the hypoxia is water logging that causes a drastic reduction in productivity in important crops. So far, a couple of studies have addressed differential expression of miRNAs under hypoxia. Using miRNA microarrays, 39 hypoxia-responsive miRNAs were discovered in maize, targeting not only mainly transcription factors as usual but also proteins involved in carbohydrate and lipid metabolism [116]. Similarly in *Arabidopsis*, 25 hypoxia-responsive miRNA families were identified using next-generation sequencing. Importantly, similar changes in miRNA expression to those caused by hypoxia were observed when a chemical treatment directed to block the normal mitochondrial respiration was applied, indicating a pivotal and active role of the mitochondria in the signal transmission during the response to oxygen deprivation [117]. The search for *cis*-elements regulating the expression of the above-mentioned miRNAs allowed the identification of motifs such as ARE (anaerobic response element), LTR, and several hormone-responsive elements (ABA, gibberellin, and ethylene) governing miRNA expression [116].

25.5.4
In Deep Water: Water Balance Stresses (Drought and Salinity) and miRNAs

The accelerated desertification of cultivable land and the climate change have brought several problems associated with water availability. Drought has the greatest effect on agriculture, causing significant losses to several economically important crops [118]. The use of different approaches has allowed the identification of several drought-induced miRNAs in *Arabidopsis* such as miR393, miR397, and miR402 [96] as well as miR167, miR168, miR171, and miR396 [112]. Conserved miRNAs, miR171 and miR393, were also induced in rice plants by drought [119], but not in *M. truncatula* [120]. These miRNAs target transcription factors and as mentioned in Section 25.4, miR393 targets TIR, an F-box protein that is a positive regulator of auxin signaling. The inhibition of TIR1, caused by increased expression of miR393, suggests an arrest of plant growth under drought stress [96, 112]. In *M. truncatula*, drought-induced miRNAs include miR398 and miR408 [120]. Together, these results reinforce the idea of overlapping function of some miRNAs in response to different conditions. In maize, miR474 was identified to be upregulated in response to drought [47], causing repression of target proline dehydrogenase gene (*PDH*) and thus favoring the accumulation of proline [47]. Proline has a protective function during drought preventing membrane damage and protein denaturation [47]. Among the *cis*-elements regulating drought-responsive miRNAs, there are MYB sites involved in drought induction (MBS) [112], as well as DREs (dehydration-responsive element)/CRT (C-repeat) in the upstream region of rice miR169g that binds the ERF/AP2 family transcription factors under drought stress [119].

Salinity has become a serious problem for plants predominantly due to intensive agricultural practices that along with climate change produce long and hotter

summers and cause decrease in rainfall. All these factors prevent the elimination of excessive salts present in soils [121]. In *Arabidopsis*, initial work showed miR393 to be strongly induced by salinity, while miR397b and miR402 were moderately induced [96]; miR156, miR159, and miR394 were shown to be specifically induced in response to salinity, while other 11 miRNAs were also induced by cold and by other abiotic stresses [112]. Thus, recent works have implicated miR156 and miR159 in other types of stresses as well (Table 25.1). In maize, the comparison of miRNA expression between two maize lines, one salt tolerant and other salt sensitive, allowed the detection of 18 miRNAs induced only in the salt-tolerant line indicating a varietal-dependent response to salt stress [122].

Some important loci-specific differences in miRNA families and their targets have been found in salinity responses. For example, from the 17 members of the miR169 family in rice, only miR169g and miR169n were induced in response to salt stress [119, 123]. Of the several transcription factors of the NF-YA family predicted as targets of miR169, only NF-YA8 was downregulated [123].

In rice, miR396 is repressed under salinity and alkaline stress [124]. In addition, *Arabidopsis* and rice transgenic plants overexpressing miR396 show a decrease in their tolerance to both stresses [124]. Also, seed germination was retarded in transgenic *Arabidopsis* plants expressing constitutively miR417, a salt-repressed miRNA, under conditions of high salinity, indicating a role of this miRNA as a negative regulator of seed germination under salinity [125], whereas plants over-expressing miR402 showed an accelerated growth under this stress condition [126].

25.6
The Strange Case of miR398: Crosstalk between miRNA-Mediated Responses to Biotic and Abiotic Stresses

One of the best-characterized miRNAs related to stress responses is miR398 that targets Cu/Zn-SOD enzymes (*CSD1* and *CSD2*), cytochrome-*c* oxidases (COX), and the chaperone CCS1 [29, 127, 128]. miR398 has been found to be repressed under some abiotic stress conditions known to induce ROS (reactive oxygen species) production in *Arabidopsis*, such as high light levels and heavy metals (Cu^{2+} and Fe^{3+}). The mRNA expression of its targets *CSD1* and *CSD2* mRNA was coordinately increased [128]. These enzymes are involved in the ROS detoxification, by converting superoxide radicals (O_2^-) to hydrogen peroxide. The expression of miR398 was also reduced after ozone fumigation, high salinity, or inoculation with avirulent strains of the bacteria PtoDC3000 [88]. Curiously, in these cases only the mRNA levels for *CSD1*, but not for *CSD2*, were inversely correlated with the expression of miR398 [88] indicating a possible specific role of these SOD enzymes in particular kinds of stresses. In addition, it was shown that transgenic *Arabidopsis* plants overexpressing a miR398-resistant version of *CSD2* present a higher accumulation of *CSD2* transcripts and in consequence are more tolerant to different oxidative agents [128]. In *Populus trichocarpa* plants treated with ABA or subjected to salt stress, miR398 is induced at early stages (first 3–4 h), but after 48 h the induction declines and is finally

Table 25.1 Commonly studied differentially expressed miRNA families under different types of stress.

Family	Target	Plant	Type	Stress	Regulation	Reference
miR156	Squamosa-promoter binding protein-like (SPL)	*A. thaliana*	A	Cold	↑	[113]
		A. thaliana	A	Hypoxia	↑	[117]
		A. thaliana	A	P limitation	↑	[104]
		P. trichocarpa	A	Cold	↓	[115]
		A. thaliana	B	Bacteria infection (Ps)	↓	[49]
		A. thaliana	B	Viral infection	↑	[73]
		Nicotiana benthamiana	B	Viral infection	↑	[159]
		P. taeda	B	Fungal infection	↓	[94]
miR159	MYB transcription factor, TCP transcription factor	*A. thaliana*	A	Cold	↑	[96]
		A. thaliana	A	Fe limitation	↑	[107]
		A. thaliana	A	Hypoxia	↑	[117]
		M. truncatula	A	Cd excess	↑	[109]
		A. thaliana	B	Bacteria infection (Ps)	↑	[49]
		P. taeda	B	Fungal infection	↓	[94]
		S. lycopersicum	B	Viral infection	↑	[75]
miR160	Auxin response factor	*P. trichocarpa*	A	Cold	↑	[115]
		A. thaliana	B	Bacteria infection (Ag)	↓	[85]
		A. thaliana	B	Bacteria infection (Ps)	↑	[48]
		A. thaliana	B	Viral infection	↑	[73]
		N. benthamiana	B	Viral infection	↑	[159]
		P. taeda	B	Fungal infection	↓	[94]
miR167	ARF	*A. thaliana*	A	Drought	↑	[112]
		A. thaliana	A	N limitation	↓	[106]
		A. thaliana	B	Bacteria infection (Ps)	↑	[48]
miR168	AGO1	*A. thaliana*	A	Cold, salinity, drought	↑	[112]
		P. trichocarpa	A	Cold	↑	[115]
		A. thaliana	B	Bacteria infection (Ps)	↓	[48]

Table 25.1 (*Continued*)

Family	Target	Plant	Type	Stress	Regulation	Reference
miR169	HAP2, NF-YA transcription factor	*A. thaliana*	A	Cold	↑	[112]
		A. thaliana	A	Fe limitation	↑	[107]
		A. thaliana	A	N limitation	↓	[102]
		A. thaliana	A	P limitation	↓	[104]
		B. distachyon	A	Cold	↑	[114]
		O. sativa	A	Drought	↑	[119]
		O. sativa	A	Salinity	↑	[123]
		A. thaliana	B	Bacteria infection (Ps)	↑	[49]
		A. thaliana	B	Viral infection	↑	[73]
		N. benthamiana	B	Viral infection	↑	[73]
miR171	Scarecrow-like transcription factor (SCL)	*A. thaliana*	A	Cold, salinity, drought	↑	[112]
		B. napus	A	Cd excess	↓	[110]
		M. truncatula	A	Cd excess	↑	[109]
		O. sativa	A	Drought	↑	[123]
		S. lycopersicum	B	Viral infection	↑	[75]
miR172	Apetala2-like transcription factor (AP2)	*A. thaliana*	A	Cold	↑	[112]
		A. thaliana	A	Fe limitation	↑	[107]
		B. dystachion	A	Cold	↑	[114]
miR393	Transport inhibitor response 1 (TIR1)/Auxin F-box (AFB)	*A. thaliana*	A	Cold, salinity	↑	[112]
		A. thaliana	A	Cold, drought, salinity	↑	[96]
		B. napus	A	Cd excess	↓	[110]
		O. sativa	A	Salinity	↓	[160]
		O. sativa	A	Drought	↑	[123]
		P. trichocarpa	A	Cold	↑	[115]
		A. thaliana	B	Bacteria infection (Ag)	↓	[85]
		A. thaliana	B	Bacteria infection (Ps)	↑	[83]
miR396	Growth regulating factor (GRF)	*A. thaliana*	A	Cold, salinity, drought	↑	[112]
		M. truncatula	A	Cd excess	↑	[109]
		P. trichocarpa	A	Cold	↑	[115]
		O. sativa	A	Salinity	↓	[124]

(*Continued*)

Table 25.1 (*Continued*)

Family	Target	Plant	Type	Stress	Regulation	Reference
		A. thaliana	B	Bacteria infection (Ps)	↑	[49]
miR397	Laccase	*A. thaliana*	A	Cold	↑	[112]
		A. thaliana	A	Cold, drought, salinity	↑	[96]
		A. thaliana	A	Cu limitation	↑	[108]
		B. dystachion	A	Cold	↑	[114]
		G. max	A	P limitation	↑	[105]
miR398	Copper superoxide dismutase (CSD), cytochrome-*c* oxidase, CCS1	*A. thaliana*	A	Cold	↑	[113]
		A. thaliana	A	N, P limitation	↓	[102]
		A. thaliana	A	Cu limitation	↓	[128]
		A. thaliana	A	P limitation	↓	[104]
		G. max	A	P limitation	↓	[105]
		M. truncatula	A	Cd excess	↓	[110]
		M. truncatula	A	Drought	↑	[120]
		A. thaliana	B	Bacteria infection (Ps)	↓	[49]
miR408	Laccase (LAC), plastocyanin-like (PCL)	*A. thaliana*	A	Cold	↑	[112]
		A. thaliana	A	Cu limitation	↑	[108]
		M. truncatula	A	Drought	↑	[120]

Type indicates type of stress (A: abiotic, B: biotic). ↑ = induced ↓ = repressed.

accumulated again over a prolonged stress (72 h). In *Arabidopsis* plants grown under the same conditions, miR398 shows an opposite response, indicating a species-specific behavior of some miRNAs in stress responses [129]. miR398 also plays an important role in response to copper stress conditions. Under low copper concentrations, it was demonstrated that the stability of *COX5b* mRNA was regulated by the action of miR398 [128, 130]. It has also been shown to be repressed in *Arabidopsis* plants growing in low nitrogen or phosphorus [102, 104], and in *M. truncatula* in response to Cd [109]. Furthermore, miR398 was induced in response to cold and sucrose in *Arabidopsis* [113, 131] and to drought in *M. truncatula* [120]. Importantly, it has been shown that miR398 can produce silencing of targets with imperfect complementarity (thus having targets that often escape traditional target prediction) and produce silencing through translational repression instead of mRNA cleavage [127, 131].

The differential expression under various conditions of miR398 and other extensively studied miRNA families is summarized in Table 25.1. As is clear from these results, many miRNAs may be playing more than one role in different types of stress.

The case of miR398 is revealing in the sense that although a clear biological role has been established for this family with an evident effect on the outcome of stress responses as in the case of ROS detoxification and copper regulation [128, 130], some aspects of its regulation are still unclear, as well as the role it may play in other types of stresses.

As we have seen in this chapter, miRNA regulation may vary in a tissue-specific [91, 105, 111], stress-specific, and species-specific manner (Table 25.1). In addition, nontraditional miRNA-mediated silencing such as translational repression through imperfect miRNA:target pairing (parameters for this type of pairing are still unknown) can occur [53, 127, 131]. And more importantly, there are multiple layers of complexity in miRNA regulation that are often difficult to assess; this complexity arises from multiple *cis*-elements that control miRNA transcription (e.g., one miRNA may be regulated by various transcription factors), as well as the multiplicity of targets for one miRNA, and multiple loci expressing related miRNA (families) and each loci perhaps under different transcriptional control. Recently, through bioinformatics, the complexity of miRNA regulatory networks was analyzed (although expression levels and *cis*-regulatory elements were not yet considered), showing a high degree of connectivity for some miRNAs [132].

Although differential expression has been the usual method for identifying biologically relevant miRNAs, all the above-mentioned factors may be affecting the outcome that these differentially expressed miRNAs may bring upon physiological processes, making it hard to differentiate truly significant stress-responsive miRNAs from those resulting from interconnectivity or pleiotropic effects. This must be taken into account when assigning a biological role to miRNAs, especially when genetic transformation strategies for stress resistance are intended.

25.7
Viva la Revolución: Using miRNA-Mediated Strategies to Develop Stress-Resistant Crops

25.7.1
Imitation of Silence: Artificial miRNAs in Plants' Genetic Transformation

As we mentioned before, one of the big drivers behind miRNA studies is the prospects that these molecules offer in genetic transformation to improve agronomic traits in plants. One way to use miRNAs in genetic transformation is through amiRNAs, which basically consist of transgenes made of endogenous miRNAs modified to silence any desired target.

amiRNAs are designed from an endogenous miRNA precursor that is used as a structural support in which the miRNA:miRNA* region is replaced with a specific miRNA complementary to the desired target sequence. The processing *in planta* of this new precursor will be the same as the endogenous one, as long as the secondary structure of the precursor is kept intact [133–135]. When designing an amiRNA, the sequence must be optimized in specificity and effectiveness; this means to select a

sequence with no potential off-targets in the desired genome and with high affinity for the target. Bioinformatics tools are available for these analyses [136]. The designed miRNA and the passenger strand are then inserted to replace the miRNA:miRNA* duplex in the endogenous miRNA precursors. The sequence can be inserted in plasmids containing the precursor through directed mutagenesis, overlapping PCR, or enzymatic digestion [136, 137]. The amiRNA transformation vector must have a termination sequence and a promoter. Inducible and constitutive promoters have shown good results in amiRNA-mediated transformation [133, 136, 138, 139]. A schematic representation of this method can be seen in Figure 25.3.

Prior to amiRNAs, various methods for plant transformation and functional gene studies based on small silencing RNAs were developed. These had some advantages over traditional methods (i.e., transposon tagging, insertional mutagenesis, and TILLING) like being able to simultaneously silence related genes and silencing genes in an inducible or tissue-specific manner [133]. These methods were based on the production of dsRNAs from a given source that produced siRNAs to silence the desired gene, for example, antisense RNA, hp-RNA, and virus-induced gene silencing (VIGS) [140, 141]. Although these tools are still widely used, they have some disadvantages regarding specificity and stability. The inserts used in these strategies are often long (>200 bp) and will not only generate a large number of siRNAs complementary to various regions of the mRNA but also possibly generate siRNAs

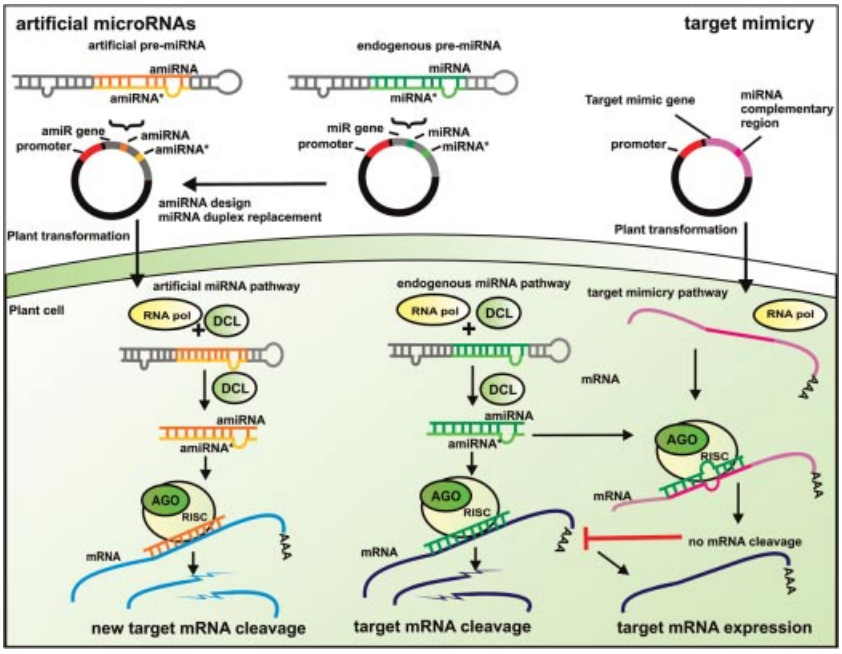

Figure 25.3 miRNA-related plant genetic transformation strategies: artificial miRNAs (amiRNAs) and target mimicry. DCL: Dicer-like; AGO: Argonaute; RISC: RNA-induced silencing complex; RNA pol: RNA polymerase.

partially complementary to undesired regions of the genome (off-targets) that are usually hard to predict. It is estimated that 50–70% of the genes in a genome could produce off -targets if used in RNA silencing strategies [137, 139, 142]. Moreover, these transgenes can be autosilenced resulting in a loss of the desired phenotype (a silenced gene) after several generations [137, 138, 143].

Successful amiRNA-mediated gene-silencing experiments have been conducted in dicotyledonous plants (*A. thaliana*, *M. truncatula*, tomato, tobacco, and soybean) [135, 144, 145], monocotyledonous plants (rice and wheat) [146, 147], mosses (*Physcomithrella patens*) [142], and even algae *(Chlamydomonas reinhardtii*) [137, 138]. They have shown to offer some advantages over other ssRNA-mediated strategies; these include (i) specificity as high as with endogenous miRNAs, (ii) absence of off targets, and (iii) absence of secondary siRNA formation or nonautonomous effects [135, 139, 144, 146, 148, 149]. They have been shown to be stable through various generations [137, 138, 146]. Also, it is possible both to generate constructs expressing multiple and unrelated amiRNAs due to their small size [66, 150] and to design amiRNAs targeting specific alleles or splice forms of a given gene [136]. It has also been proposed that amiRNAs pose fewer biosafety or environmental problems when applied to agriculture than other strategies [23, 68].

There are, however, some difficulties in working with amiRNAs. The success rate of amiRNA, inferred from various published studies, may vary from 75 to 90 % [66, 67, 139, 144, 148, 151]. The reasons why some genes cannot be silenced through amiRNAs are not yet clear. A possible explanation may be the difficult accessibility of the target mRNA to the RISC, which could be overcome in amiRNA design by thermodynamically modeling mRNA accessibility [152]. Also, amiRNA activity may be impaired by a negative regulation mechanism where the artificially produced silencing is compensated by an increase in the transcription rates of the targeted mRNA, though this has not been proved yet [136].

Along with amiRNAs, other novel miRNA-related strategies have been developed to improve plant genetic transformation. Artificial ta-siRNAs have been designed in a similar way to that of amiRNAs by replacing the siRNA region in the TASc1 ta-siRNA gene (which requires miR173-mediated cleavage to produce siRNAs) with sequences complementary to new desired targets, producing effective silencing [153]. There is also a technique to improve amiRNA's effectiveness by designing them from long primary miRNAs instead of pre-miRNAs [154].

Likewise, another promising strategy is target mimicry, where a noncleavable mRNA is inserted into the plant to interact in a non-silencing manner with a miRNA, thus inhibiting its activity (Figure 25.3). This strategy would allow modulation of the expression level of endogenous miRNAs to achieve a greater expression of genes normally regulated by miRNAs. Although this strategy has not being extensively exploited yet, recently a valuable large-scale collection of knockdowns for *Arabidopsis* was made comprising most known miRNA families in this plant [23, 103, 155].

So far, amiRNAs and related strategies have been used in a few model and crop plant species, particularly *Arabidopsis*, where there are even ongoing efforts to have amiRNA libraries covering all annotated genes [133]. In a short time, the techniques have spread to a larger number of plant species and are rapidly becoming reliable

tools for functional gene analysis [23, 145, 156]. amiRNAs are still a technique used mostly for research purposes, and they have not been yet extensively employed in crops to improve agronomical traits such as stress resistance. The potential for the technique is great, and probably we are not so far from having commercially available crop varieties transformed with amiRNAs.

25.7.2 Biotic Stress Resistance

From early in their development, it was foreseen that one of the greatest potentials for amiRNAs would be to produce plants with enhanced stress resistance, especially for antiviral defense. Unlike other types of stresses, viral infection has been already addressed through miRNA-mediated technologies. It has been demonstrated using amiRNAs that the miRNA pathway in plants can function in antiviral defense. Transgenic *Arabidopsis* and tobacco plants have been transformed to develop resistance against TMV (turnip mosaic virus), TuMV (turnip yellow mosaic virus), and CMV (cucumber mosaic virus) by inserting amiRNAS complementary into viral genome regions [66–68, 150]. These efforts have revealed resistance at cellular level, inheritable, more efficient than siRNA-mediated strategies, and successful in blocking viral replication and movement [66, 67]. Furthermore, it has been possible to create transgenic plants with dimeric amiRNAs directed against different viruses resulting in plants with a wide spectrum of viral resistance [66, 150].

There are, however, some difficulties to overcome when considering a widespread use of amiRNAs in antiviral defense. The presence in many viruses of silencing suppressors that interfere with the miRNA machinery poses an obstacle that could be solved by designing amiRNAs directed against silencing suppressors [66, 67]. The most important objection comes from the fact that viral genomes evolve a lot faster than plant miRNAs and can evade miRNA targeting by mutating the targeted region [150, 157]. A solution has been proposed that mainly consists of a "polymeric strategy" where transgenic plants have amiRNAs directed against several viral regions, preferentially conserved [66, 68, 150]. The effectiveness of such strategy has not being completely tested yet.

The use of amiRNAs to develop resistance against other pathogens can be slightly more difficult than with viruses since there is no direct interaction with the pathogen's nucleic acids. Thus, efforts for amiRNA-mediated antipathogen resistance should aim at modifying the plant defense responses through miRNA regulation, which could also work as an indirect way for antiviral defense.

Our present knowledge of miRNA roles in antipathogen defense points to two principal modes of regulating plant defense (Figure 25.1): the induction of miRNAs targeting negative regulators of plant defense and the repression of miRNAs targeting positive regulators of plant defense. These two strategies could be artificially reproduced in plants in various ways: (i) by overexpressing pathogen-induced miRNAs, (ii) using amiRNAs directed against negative regulators of plant defense, and (iii) through target mimicry using mRNAs binding to miRNAs cleaving positive regulators of plant defense.

Overexpression of pathogen-induced miRNAs, as in the case of PtoDC3000-responsive miR393 in *Arabidopsis*, has proven to increase resistance to bacterial infection; however, some developmental defects have been observed [83] that are undesirable when trying to take this strategy to crops. Additional attempts to improve antipathogen resistance by miRNA overexpression are scarce.

In the case of amiRNAs and target mimicry, with these strategies, it could be possible to accentuate existing miRNA-mediated responses in plants, for example, by introducing new amiRNAs in plants to target auxin response factors coupled to pathogen-inducible promoters (in the same way as miR393 and miR160 act) or using pathogen-inducible target mimicry to further repress the activity of miRNAs such as miR825. The techniques could also be used to develop novel resistance mechanisms by insertion of amiRNAs targeting negative regulators of plant defense, which are not normally under miRNA regulation. However, given the mentioned difficulties in assessing miRNA roles in stress (see Section 25.6), these approaches must be coupled to a complete understanding of miRNA-regulated networks.

25.7.3
Abiotic Stress Resistance

By understanding the complex regulatory circuits involved in miRNA-mediated gene expression, it is possible to think of ways to manipulate these circuits to produce plants with an increased tolerance to a particular kind of abiotic stress. There are some examples where the manipulation of miRNA regulation has produced desired effects in plants. In one case, overexpression of a mimic *CSD* gene, resistant to miR398 degradation, produced plants more tolerant to higher levels of light and heavy metals [128]. The potential to manipulate miRNAs to obtain desired traits is great, although it should be considered carefully.

One possibility to modify miRNA-mediated responses to abiotic stress, which has already been explored, is to produce transgenic plants overexpressing miRNAs to increase the degradation of their target genes; varying results have been obtained with this approach. For example, overexpression of miR402 (a salinity- and drought-induced miRNA family) produced an accelerated seedling growth under salinity stress condition [126], which would be a desired trait for crops to grow in difficult soils. However, the overexpression of particular miRNAs could produce undesired effects. For example, transgenic plants with constitutive expression of miR399 (normally induced under P starvation) displayed an increased accumulation of P without great adverse effects [99], whereas in other cases, plants overexpressing miR399 showed toxicity symptoms produced by the uncontrolled P uptake and by a pronounced P translocation from roots to shoots [100].

Caution must also be exerted when trying to translate results from research in model species into crops due to the mostly unknown species-specific effects on miRNA responses despite the high conservation of some miRNA families. For example, in one case, the overexpression of conserved miR396 in rice produced a

reduced tolerance to salinity and alkali conditions [124], while the overexpression of this miRNA in *Arabidopsis* increased the tolerance to drought and other abiotic stresses [158].

Engineering new miRNA pathways using amiRNAs and target mimicry may also be possible as our knowledge of abiotic stress responses increases.

25.8 Conclusions and Perspectives

The use of miRNAs as a tool for plant genetic engineering is exciting and likely to be highly rewarding, and there are surely many ongoing efforts to exploit the miRNA pathway for crop improvement. However, there are still large gaps in our knowledge of miRNA-mediated regulation as is evident from the increasing inventory of differentially expressed miRNA under stress conditions for which a clear physiological outcome in stress responses has not been established.

The complexity of the regulatory networks mediated by miRNAs in plants is perhaps still not fully perceived. There is undoubtedly a need to establish comprehensible links between the transcriptome, the microme (microRNome or microtranscriptome), the degradome, and the proteome (including regulatory *cis*-elements) in order to conceive the modification or creation of miRNA pathways without adverse physiological consequences. Efforts should thus be addressed to model gene regulatory networks incorporating large sets of biological data; this is now more feasible than once expected due to the significant improvements in sequencing technologies, bioinformatics, and systems biology.

The expansion of miRNA research to plants different from the traditional models may also be essential to future miRNA genetic transformation given the species-specific differences in conserved miRNA regulation and the unexplored potential of phylogenetically restricted miRNA families. This is particularly true for stress responses where such variability is displayed among relatively narrow phylogenetic ranges (e.g., the wide range of environmental adaptations among the *Poaceae*). The results shown by miRNA research on relatively new models for specific processes (such as soybean and *M. truncatula* for root symbiosis studies and *B. dystachion* for cold responses) confirm the importance of this type of studies, and it is our expectation that this field will grow to include less explored plants such as mangroves for salinity and drowning tolerance and cassava for drought tolerance.

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References

1 Napoli, C., Lemieux, C., and Jorgensen, R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell*, **2** (4), 279–289.

2 Van Der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N., and Stuitje, A.R. (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell*, **2** (4), 291–299.

3 Guo, S. and Kemphues, K.J. (1995) Par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell*, **81** (4), 611–620.

4 Cogoni, C., Irelan, J.T., Schumacher, M., Schmidhauser, T.J., Selker, E.U., and Macino, G. (1996) Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA–DNA interactions or DNA methylation. *Eur. Mol. Biol. Org. J.*, **15** (12), 3153–3163.

5 Voinnet, O. and Baulcombe, D.C. (1997) Systemic signalling in gene silencing. *Nature*, **389** (6651), 553.

6 Kidner, C. and Martienssen, R. (2005) The developmental role of microRNA in plants. *Curr. Opin. Plant Biol.*, **8** (1), 38–44.

7 Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature*, **431** (7006), 343–349.

8 Lippman, Z. and Martienssen, R. (2004) The role of RNA interference in heterochromatic silencing. *Nature*, **431** (7006), 364–370.

9 Filipowicz, W., Jaskiewicz, L., Kolb, F.A., and Pillai, R.S. (2005) Post-transcriptional gene silencing by siRNAs and miRNAs. *Curr. Opin. Struct. Biol.*, **15** (3), 331–341.

10 Tang, G., Reinhart, B.J., Bartel, D.P., and Zamore, D. (2003) A biochemical framework for RNA silencing in plants. *Genes Dev.*, **2**, 49–63.

11 Bartel, D. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116** (2), 281–297.

12 Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell*, **121** (2), 207–221.

13 Yoshikawa, M., Peragine, A., Park, M.Y., and Poethig, R.S. (2005) A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Gene Dev.*, **19** (18), 2164–2175.

14 Borsani, O., Zhu, J., Verslues, E., and Sunkar, R. (2005) Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell*, **123** (7), 1279–1291.

15 Matzke, M.A. and Birchler, J.A. (2005) RNAi-mediated pathways in the nucleus. *Nat. Rev. Genet.*, **6** (1), 24–35.

16 Matzke, M., Kanno, T., Daxinger, L., Huettel, B., and Matzke, A.J.M. (2009) RNA-mediated chromatin-based silencing in plants. *Curr. Opin. Cell Biol.*, **21** (3), 367–376.

17 Katiyar-Agarwal, S., Gao, S., Vivian-Smith, A., and Jin, H. (2007) A novel class of bacteria-induced small RNAs in *Arabidopsis*. *Genes Dev.*, **21** (23), 3123–3134.

18 Wu, L., Zhou, H., Zhang, Q., Zhang, J., Ni, F., Liu, C., and Qi, Y. (2010) DNA methylation mediated by a microRNA pathway. *Mol. Cell*, **38** (3), 465–475.

19 Mochizuki, K. and Gorovsky, M.A. (2004) Conjugation-specific small RNAs in Tetrahymena have predicted properties of scan (scn) RNAs involved in genome rearrangement. *Genes Dev.*, **18** (17), 2068–2073.

20 Hartig, J.V., Tomari, Y., and Förstemann, K. (2007) piRNAs: the ancient hunters of genome invaders. *Genes Dev.*, **21** (14), 1707–1713.

21 Klattenhoff, C. and Theurkauf, W. (2008) Biogenesis and germline functions of piRNAs. *Development*, **135** (1), 3–9.

22 Vaishnaw, A.K., Gollob, J., Gamba-Vitalo, C., Hutabarat, R., Sah, D., Meyers, R., De Fougerolles, T., and Maraganore, J. (2010) A status report on RNAi therapeutics. *Silence*, **1** (1), 14.

23 Liu, Q. and Chen, Y.-Q. (2010) A new mechanism in plant engineering: the potential roles of microRNAs in molecular breeding for crop improvement. *Biotechnol. Adv.*, **28** (3), 301–307.

24 Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, **75** (5), 843–854.

25 Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391** (6669), 806–811.

26 Montgomery, M.K., Xu, S., and Fire, A. (1998) RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA*, **95** (26), 15502–15507.

27 Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D. (2002) MicroRNAs in plants. *Genes Dev.*, **16**, 1616–1626.

28 Llave, C., Kasschau, K.D., Rector, M.A., and Carrington, J.C. (2002) Endogenous and silencing-associated small RNAs in plants. *Plant Cell*, **14** (7), 1605.

29 Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006) MicroRNAS and their regulatory roles in plants. *Annu. Rev. Plant Biol.*, **57**, 19–53.

30 Zhu, J.K. (2008) Reconstituting plant miRNA biogenesis. *Proc. Natl. Acad. Sci. USA*, **105** (29), 9851.

31 Millar, A.A. and Waterhouse, M. (2005) Plant and animal microRNAs: similarities and differences. *Funct. Integr. Genomics*, **5** (3), 129–135.

32 Brennecke, J., Stark, A., Russell, R.B., and Cohen, S.M. (2005) Principles of microRNA-target recognition. *PLoS Biology*, **3** (3), e85.

33 Griffiths-Jones, S. (2010) miRBase: microRNA sequences and annotation. Wiley Online Library, Chapter 12, Unit 12.9.1–10 http://www.mirbase.org/ (5 Nov. 2010).

34 Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003) Control of leaf morphogenesis by microRNAs. *Nature*, **425** (6955), 257–263.

35 Wu, G. and Poethig, R.S. (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. *Development*, **133** (18), 3539–3547.

36 Aukerman, M.J. and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. *Plant Cell*, **15** (11), 2730–2741.

37 Kutter, C., Schöb, H., Stadler, M., Meins, F., and Si-Ammour, A. (2007) MicroRNA-mediated regulation of stomatal development in *Arabidopsis*. *Plant Cell*, **19** (8), 2417–2429.

38 Shukla, L.I., Chinnusamy, V., and Sunkar, R. (2008) The role of microRNAs and other endogenous small RNAs in plant stress responses. *BBA Gene Regul. Mech.*, **1779** (11), 743–748.

39 Ruiz-Ferrer, V. and Voinnet, O. (2009) Roles of plant small RNAs in biotic stress responses. *Annu. Rev. Plant Biol.*, **60**, 485–510.

40 Brennecke, J., Hipfner, D.R., Stark, A., Russell, R.B., and Cohen, S.M. (2003) Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. *Cell*, **113** (1), 25–36.

41 Ruvkun, G., Wightman, B., and Ha, I. (2004) The 20 years it took to recognize the importance of tiny RNAs. *Cell*, **116** (2 Suppl), S93–S96, 2 p. following S96.

42 Zhang, B., Pan, X., Wang, Q., Cobb, G.P., and Anderson, T.A. (2006) Computational identification of microRNAs and their targets. *Comput. Biol. Chem.*, **30** (6), 395–407.

43 Unver, T., Namuth-covert, D.M., and Budak, H. (2009) Review of current methodological approaches for characterizing microRNAs in plants. *Int. J. Plant Genomics*, **2009**.

44 ILLUMINA, Inc . (2009) http://www.illumina.com/ (5Nov. 2010).

45 Nobuta, K., McCormick, K., Nakano, M., and Meyers, B.C. (2009) Bioinformatics analysis of small RNAs in plants using next generation sequencing technologies. *Methods Mol. Biol.*, **592** (1), 89–106.

46 Willenbrock, H., Salomon, J., Søkilde, R., Barken, K.B., Hansen, T.N., Nielsen, F.C., Møller, S., and Litman, T. (2009) Quantitative miRNA expression analysis: comparing microarrays with next-generation sequencing. *RNA*, **15** (11), 2028.

47 Wei, B., Cai, T., Zhang, R., and Li, A. (2009) Novel microRNAs uncovered by deep sequencing of small RNA transcriptomes in bread wheat (*Triticum aestivum* L.) and *Brachypodium distachyon* (L.) Beauv. *Funct. Integr. Genomics.*, **9** (4), 499–511.

48 Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A., Law, T.F., Grant, S.R., Dangl, J.L., and Carrington, J.C. (2007) High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of MIRNA genes. *PLoS ONE*, **2** (2), e219.

49 Li, Y., Zhang, Q.Q., Zhang, J., Wu, L., Qi, Y., and Zhou, J.M. (2010) Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiol.*, **152** (4), 2222.

50 Barbato, C., Arisi, I., Frizzo, M.E., Brandi, R., Da Sacco, L., and Masotti, A. (2009) Computational challenges in miRNA target predictions: to be or not to be a true target? *J. Biomed. Biotechnol.*, **2009**, 803069.

51 Mazière, P. and Enright, A.J. (2007) Prediction of microRNA targets. *Drug Discov. Today*, **12** (11–12), 452–458.

52 Brodersen, P. and Voinnet, O. (2009) Revisiting the principles of microRNA target recognition and mode of action. *Nat. Rev. Mol. Cell Biol.*, **10** (2), 141–148.

53 Yu, B. and Wang, H. (2010) Translational inhibition by microRNAs in plants. *Prog. Mol. Subcell. Biol.*, **50**, 41–57.

54 Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. (2002) Cleavage of scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science*, **297** (5589), 2053–2056.

55 German, M.A., Luo, S., Schroth, G., Meyers, B.C., and Green, J. (2009) Construction of parallel analysis of RNA ends (PARE) libraries for the study of cleaved miRNA targets and the RNA degradome. *Nat. Protoc.*, **4** (3), 356–362.

56 Addo-Quaye, C., Snyder, J.A., Park, Y.B., Li, Y.F., Sunkar, R., and Axtell, M.J. (2009) Sliced microRNA targets and precise loop-first processing of MIR319 hairpins revealed by analysis of the *Physcomitrella patens* degradome. *RNA*, **15** (12), 2112.

57 Meng, Y., Gou, L., Chen, D., Wu, P., and Chen, M. (2010) High-throughput degradome sequencing can be used to gain insights into microRNA precursor metabolism. *J. Exp. Bot.*, **61** (14), 3833–3837.

58 Molnar, A., Melnyk, C.W., Bassett, A., Hardcastle, T.J., Dunn, R., and Baulcombe, D.C. (2010) Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science*, **328** (5980), 872–875.

59 Palauqui, J.C., Elmayan, T., Pollien, J.M., and Vaucheret, H. (1997) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *Eur. Mol. Biol. Org. J.*, **16** (15), 4738–4745.

60 Roth, B.M., Pruss, G.J., and Vance, V.B. (2004) Plant viral suppressors of RNA silencing. *Virus Res.*, **102** (1), 97–108.

61 Llave, C. (2004) MicroRNAs: more than a role in plant development? *Mol. Plant Pathol.*, **5**, 361–366.

62 Dunoyer, P., Lecellier, C.H., Parizotto, E.A., and Himber, C. (2004) Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell*, **16**, 1235–1250.

63 Mourrain, P., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., Rémoué, K., Sanial, M., Vo, T.A., and Vaucheret, H. (2000)

Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell*, **101** (5), 533–542.

64 Lecellier, C.-H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saïb, A., and Voinnet, O. (2005) A cellular microRNA mediates antiviral defense in human cells. *Science*, **308** (5721), 557–560.

65 Pérez-Quintero, Á.L., Neme, R., Zapata, A., and López, C. (2010) Plant microRNAs and their role in defense against viruses: a bioinformatics approach. *BMC Plant Biol.*, **10** (1), 138.

66 Niu, Q.-W., Lin, S.-S., Reyes, J.L., Chen, K.-C., Wu, H.-W., Yeh, S.-D., and Chua, N.-H. (2006) Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat. Biotechnol.*, **24** (11), 1420–1429.

67 Qu, J., Ye, J., and Fang, R. (2007) Artificial microRNA-mediated virus resistance in plants. *J. Virol.*, **81** (12), 6690–6699.

68 Duan, C.-G., Wang, C.-H., Fang, R.-X., and Guo, H.-S. (2008) Artificial microRNAs highly accessible to targets confer efficient virus resistance in plants. *J. Virol.*, **82** (22), 11084–11095.

69 Boss, I.W. and Renne, R. (2010) Viral miRNAs: tools for immune evasion. *Curr. Opin. Microbiol.*, **13** (4), 540–545.

70 Bennasser, Y., Le, S.-Y., Yeung, M.L., and Jeang, K.-T. (2004) HIV-1 encoded candidate micro-RNAs and their cellular targets. *Retrovirology*, **1** (1), 43.

71 Moissiard, G. and Voinnet, O. (2006) RNA silencing of host transcripts by cauliflower mosaic virus requires coordinated action of the four *Arabidopsis* Dicer-like proteins. *Proc. Natl. Acad. Sci. USA*, **103** (51), 19593–19598.

72 Naqvi, A.R., Choudhury, N.R., Haq, Q.M.R., and Mukherjee, S.K. (2008) MicroRNAs as biomarkers in tomato leaf curl virus (ToLCV) disease. *Nucleic Acids Symp. Ser.* **52**, 507–508.

73 Bazzini, A.A., Hopp, H.E., Beachy, R.N., and Asurmendi, S. (2007) Infection and coaccumulation of tobacco mosaic virus proteins alter microRNA levels, correlating with symptom and plant development. *Proc. Natl. Acad. Sci. USA*, **104** (29), 12157–12162.

74 Bazzini, A.A., Almasia, N.I., Manacorda, C.A., Mongelli, V.C., Conti, G., Maroniche, G.A., Rodriguez, M.C., Distéfano, A.J., Hopp, H.E., del Vas, M., and Asurmendi, S. (2009) Virus infection elevates transcriptional activity of miR164a promoter in plants. *BMC Plant Biol.*, **9**, 152.

75 Feng, J., Wang, K., Liu, X., Chen, S., and Chen, J. (2009) The quantification of tomato microRNAs response to viral infection by stem-loop real-time RT-PCR. *Gene*, **437** (1–2), 14–21.

76 He, X.-F., Fang, Y.-Y., Feng, L., and Guo, H.-S. (2008) Characterization of conserved and novel microRNAs their targets, including a TuMV-induced TIR-NBS-LRR class R gene-derived novel miRNA in *Brassica*. *FEBS Lett.*, **582** (16), 2445–2452.

77 Lewsey, M., Robertson, F.C., Canto, T., Palukaitis, P., and Carr, J. (2007) Selective targeting of miRNA-regulated plant development by a viral counter-silencing protein. *Plant J.*, **50** (2), 240–252.

78 Glick, E., Zrachya, A., Levy, Y., Mett, A., Gidoni, D., Belausov, E., Citovsky, V., and Gafni, Y. (2008) Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. *Proc. Natl. Acad. Sci. USA*, **105** (1), 157–161.

79 Omarov, R., Sparks, K., Smith, L., Zindovic, J., and Scholthof, H.B. (2006) Biological relevance of a stable biochemical interaction between the tombusvirus-encoded P19 and short interfering RNAs. *J. Virol.*, **80** (6), 3000–3008.

80 Pantaleo, V., Szittya, G., and Burgyán, J. (2007) Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. *J. Virol.*, **81** (8), 3797–3806.

81 Díaz-Pendón, J.A. and Ding, S.-W. (2008) Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annu. Rev. Phytopathol.*, **46** (1), 303–326.

82 Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006) Host–microbe interactions: shaping the evolution of the plant immune response. *Cell*, **124**, 803–814.

83 Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D.G. (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science*, **312** (5772), 436–439.

84 Navarro, L., Jay, F., Nomura, K., He, S.Y., and Voinnet, O. (2008) Suppression of the microRNA pathway by bacterial effector proteins. *Science*, **321** (5891), 964–967.

85 Pruss, G.J., Nester, E.W., and Vance, V. (2008) Infiltration with *Agrobacterium tumefaciens* induces host defense and development-dependent responses in the infiltrated zone. *Mol. Plant Microbe Interact.*, **21** (12), 1528–1538.

86 Katiyar-Agarwal, S. and Jin, H. (2010) Role of small RNAs in host–microbe interactions. *Annu. Rev. Phytopathol.*, **48** (1), 225.

87 Jin, H. (2008) Endogenous small RNAs and antibacterial immunity in plants. *FEBS Lett.*, **582** (18), 2679–2684.

88 Jagadeeswaran, G., Saini, A., and Sunkar, R. (2009) Biotic and abiotic stress down-regulate miR398 expression in *Arabidopsis*. *Planta*, **229** (4), 1009–1014.

89 Jay, F., Renou, J.-P., Voinnet, O., and Navarro, L. (2010) Biotic stress-associated microRNAs: identification, detection, regulation, and functional analysis. *Methods Mol. Biol.*, **592**, 183–202.

90 Katiyar-Agarwal, S., Morgan, R., Dahlbeck, D., Borsani, O., Villegas, A., Zhu, J.-K., Staskawicz, B.J., and Jin, H. (2006) A pathogen-inducible endogenous siRNA in plant immunity. *Proc. Natl. Acad. Sci. USA*, **103** (47), 18002–18007.

91 Combier, J.-P., Frugier, F., De Billy, F., Boualem, A., El-Yahyaoui, F., Moreau, S., Vernié, T., Ott, T., Gamas, P., Crespi, M., and Niebel, A. (2006) MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. *Genes Dev.*, **20** (22), 3084–3088.

92 Subramanian, S., Fu, Y., Sunkar, R., Barbazuk, W.B., Zhu, J.-K., and Yu, O. (2008) Novel and nodulation-regulated microRNAs in soybean roots. *BMC Genomics*, **9** (1), 160.

93 Wang, Y., Li, P., Cao, X., Wang, X., Zhang, A., and Li, X. (2009) Identification and expression analysis of miRNAs from nitrogen-fixing soybean nodules. *Biochem. Biophys. Res. Commun.*, **378** (4), 799–803.

94 Lu, S., Sun, Y.-H., Amerson, H., and Chiang, V.L. (2007) MicroRNAs in loblolly pine (*Pinus taeda* L.) and their association with fusiform rust gall development. *Plant J.*, **51** (6), 1077–1098.

95 Liu, T.-Y., Chang, C.-Y., and Chiou, T.-J. (2009) The long-distance signaling of mineral macronutrients. *Curr. Opin. Plant Biol.*, **12** (3), 312–319.

96 Sunkar, R. and Zhu, J. (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Science*, **16**, 2001–2019.

97 Bari, R., Datt Pant, B., Stitt, M., and Scheible, W.-R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Phys.*, **141** (3), 988–999.

98 Aung, K., Lin, S.-I., Wu, C.-C., Huang, Y.-T., Su, C.-L., and Chiou, T.-J. (2006) Pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Phys.*, **141** (3), 1000–1011.

99 Fujii, H., Chiou, T.-J., Lin, S.-I., Aung, K., and Zhu, J.-K. (2005) A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr. Biol.*, **15** (22), 2038–2043.

100 Chiou, T.-J., Aung, K., Lin, S.-I., Wu, C.-C., Chiang, S.-F., and Su, C.-L. (2006) Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell*, **18** (2), 412–421.

101 Lin, S.-I., Chiang, S.-F., Lin, W.-Y., Chen, J.-W., Tseng, C.-Y., Wu, P.-C., and Chiou, T.-J. (2008) Regulatory network of microRNA399 and PHO2 by systemic signaling. *Plant Phys.*, **147** (2), 732–746.

102 Pant, B.D., Musialak-Lange, M., Nuc, P., May, P., Buhtz, A., Kehr, J., Walther, D., and Scheible, W.-R. (2009) Identification

of nutrient-responsive *Arabidopsis* and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. *Plant Phys.*, **150** (3), 1541–1555.

103 Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., García, J.A., and Paz-Ares, J. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat. Genet.*, **39** (8), 1033–1037.

104 Hsieh, L.-C., Lin, S.-I., Shih, A.C.-C., Chen, J.-W., Lin, W.-Y., Tseng, C.-Y., Li, W.-H., and Chiou, T.-J. (2009) Uncovering small RNA-mediated responses to phosphate deficiency in *Arabidopsis* by deep sequencing. *Plant Phys.*, **151** (128), 2120–2132.

105 Zeng, H.Q., Zhu, Y.Y., Huang, S.Q., and Yang, Z.M. (2010) Analysis of phosphorus-deficient responsive miRNAs and *cis*-elements from soybean (*Glycine max* L.). *J. Plant Phys.*, **167** (15), 1289–1297.

106 Gifford, M.L., Dean, A., Gutierrez, R.A., Coruzzi, G.M., and Birnbaum, K.D. (2008) Cell-specific nitrogen responses mediate developmental plasticity. *Proc. Natl. Acad. Sci. USA*, **105** (2), 803–808.

107 Kong, W.W. and Yang, Z.M. (2010) Identification of iron-deficiency responsive microRNA genes and *cis*-elements in *Arabidopsis*. *Plant Phys. Biochem.*, **48** (2–3), 153–159.

108 Abdel-Ghany, S.E., and Pilon, M. (2008) MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in *Arabidopsis*. *J. Biol. Chem.*, **283** (23), 15932–15945.

109 Zhou, Z.S., Huang, S.Q., and Yang, Z.M. (2008) Bioinformatic identification and expression analysis of new microRNAs from *Medicago truncatula*. *Biochem. Biophys. Res. Commun.*, **374** (3), 538–542.

110 Xie, F.L., Huang, S.Q., Guo, K., Xiang, A.L., Zhu, Y.Y., Nie, L., and Yang, Z.M. (2007) Computational identification of novel microRNAs and targets in *Brassica napus*. *Biotechnology*, **581**, 1464–1474.

111 Huang, S.Q., Peng, J., Qiu, C.X., and Yang, Z.M. (2009) Heavy metal-regulated new microRNAs from rice. *J. Inorg. Biochem.*, **103** (2), 282–287.

112 Liu, H.-H., Tian, X.I.N., Li, Y.-J., Wu, C.-A., and Zheng, C.-C. (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA*, **14**, 836–843.

113 Zhou, X., Wang, G., Sutoh, K., Zhu, J.-K., and Zhang, W. (2008) Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochim. Biophys. Acta*, **1779** (11), 780–788.

114 Zhang, J., Xu, Y., Huan, Q., and Chong, K. (2009) Deep sequencing of Brachypodium small RNAs at the global genome level identifies microRNAs involved in cold stress response. *BMC Genomics*, **10**, 449.

115 Lu, S., Sun, Y.-H., and Chiang, V.L. (2008) Stress-responsive microRNAs in *Populus*. *Plant J.*, **55** (1), 131–151.

116 Zhang, Z., Wei, L., Zou, X., Tao, Y., Liu, Z., and Zheng, Y. (2008) Submergence-responsive microRNAs are potentially involved in the regulation of morphological and metabolic adaptations in maize root cells. *Ann. Bot.*, **102** (4), 509–519.

117 Moldovan, D., Spriggs, A., Yang, J., Pogson, B.J., Dennis, E.S., and Wilson, I.W. (2010) Hypoxia-responsive microRNAs and trans-acting small interfering RNAs in *Arabidopsis*. *J. Exp. Bot.*, **61** (1), 165–177.

118 Vinocur, B. and Altman, A. (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr. Opin. Biotechnol.*, **16** (2), 123–132.

119 Zhao, B., Liang, R., Ge, L., Li, W., Xiao, H., Lin, H., Ruan, K., and Jin, Y. (2007) Identification of drought-induced microRNAs in rice. *Biochem. Biophys. Res. Commun.*, **354** (2), 585–590.

120 Trindade, I., Capitão, C., Dalmay, T., Fevereiro, M.P., and Santos, D.M.D. (2010) miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. *Planta*, **231** (3), 705–716.

121 Yamaguchi, T. and Blumwald, E. (2005) Developing salt-tolerant crop plants: challenges and opportunities. *Trends Plant Sci.*, **10** (12), 615–620.

122 Ding, D., Zhang, L., Wang, H., Liu, Z., Zhang, Z., and Zheng, Y. (2009) Differential expression of miRNAs in response to salt stress in maize roots. *Ann. Bot.*, **103** (1), 29–38.

123 Zhao, B., Ge, L., Liang, R., Li, W., Ruan, K., Lin, H., and Jin, Y. (2009) Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. *BMC Mol. Biol.*, **10**, 29.

124 Gao, P., Bai, X., Yang, L., and Lv, D. (2010) Over-expression of osa-MIR396c decreases salt and alkali stress tolerance. *Planta*, **231**, 991–1001.

125 Jung, H.J. and Kang, H. (2007) Expression and functional analyses of microRNA417 in *Arabidopsis thaliana* under stress conditions. *Plant Physiol. Biochem.*, **45** (10–11), 805–811.

126 Kim, J.Y., Kwak, K.J., Jung, H.J., Lee, H.J., and Kang, H. (2010) MicroRNA402 affects seed germination of *Arabidopsis thaliana* under stress conditions via targeting DEMETER-LIKE Protein3 mRNA. *Plant Cell Physiol.*, **51** (6), 1079–1083.

127 Bouché, N. (2010) New insights into miR398 functions in *Arabidopsis*. *Plant Signal. Behav.*, **5** (6).

128 Sunkar, R., Kapoor, A., and Zhu, J.-K. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell*, **18** (8), 2051–2065.

129 Jia, X., Wang, W., Ren, L., Chen, Q., Mendu, V., and Willcut, B. (2009) Differential and dynamic regulation of miR398 in response to ABA and salt stress in *Populus tremula* and *Arabidopsis thaliana*. *Plant Mol. Biol.*, **71** (1–2), 51–59.

130 Yamasaki, H., Abdel-Ghany, S.E., Cohu, C.M., Kobayashi, Y., Shikanai, T., and Pilon, M. (2007) Regulation of copper homeostasis by micro-RNA in *Arabidopsis*. *J. Biol. Chem.*, **282** (22), 16369–16378.

131 Dugas, D. and Bartel, B. (2008) Sucrose induction of *Arabidopsis* miR398 represses two Cu/Zn superoxide dismutases. *Plant Mol. Biol.*, **67** (4), 403–417.

132 MacLean, D., Elina, N., Havecker, E.R., Heimstaedt, S.B., Studholme, D.J., and Baulcombe, D.C. (2010) Evidence for large complex networks of plant short silencing RNAs. *PloS One*, **5** (3), e9901.

133 Ossowski, S., Schwab, R., and Weigel, D. (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant J.*, **53** (4), 674–690.

134 Park, W., Zhai, J., and Lee, J.Y. (2009) Highly efficient gene silencing using perfect complementary artificial miRNA targeting AP1 or heteromeric artificial miRNA targeting AP1 and CAL genes. *Plant Cell Rep.*, **28** (3), 469–480.

135 Parizotto, E.A., Dunoyer, P., Rahm, N., Himber, C., and Voinnet, O. (2004) *In vivo* investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. *Genes Dev.*, **18**, 2237–2242.

136 Schwab, R., Ossowski, S., Warthmann, N., and Weigel, D. (2010) Directed gene silencing with artificial microRNAs. *Methods Mol. Biol.*, **592**, 71.

137 Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., and Baulcombe, D. (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *Plant J.*, **58** (1), 1–10.

138 Zhao, T., Wang, W., Bai, X., and Qi, Y. (2009) Gene silencing by artificial microRNAs in *Chlamydomonas*. *Plant J.*, **58** (1), 157–164.

139 Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. *Plant Cell*, **18**, 1121–1133.

140 Watson, J.M., Fusaro, A.F., Wang, M., and Waterhouse, M. (2005) RNA silencing platforms in plants. *FEBS Lett.*, **579** (26), 5982–5987.

141 Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., and Baulcombe, D.C. (2003) Virus-induced gene silencing in plants. *Methods*, **30** (4), 296–303.

142 Khraiwesh, B., Ossowski, S., Weigel, D., Reski, R., and Frank, W. (2008) Specific gene silencing by artificial microRNAs in

Physcomitrella patens: an alternative to targeted gene knockouts. *Plant Physiol.*, **148** (2), 684–693.

143 McGinnis, K., Chandler, V., Cone, K., Kaeppler, H., Kaeppler, S., Kerschen, A., Pikaard, C., Richards, E., Sidorenko, L., and Smith, T. (2005) Transgene-induced RNA interference as a tool for plant functional genomics. *Methods Enzymol.*, **392**, 1–24.

144 Alvarez, J.P., Pekker, I., Goldshmidt, A., Blum, E., Amsellem, Z., and Eshed, Y. (2006) Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. *Regulation*, **18**, 1134–1151.

145 Melito, S., Heuberger, A.L., Cook, D., Diers, B.W., MacGuidwin, A.E., and Bent, A.F. (2010) A nematode demographics assay in transgenic roots reveals no significant impacts of the Rhg1 locus LRR-Kinase on soybean cyst nematode resistance. *BMC Plant Biol.*, **10**, 104.

146 Warthmann, N., Chen, H., Ossowski, S., Weigel, D., and Hervé, P. (2008) Highly specific gene silencing by artificial miRNAs in rice. *PLoS One*, **3** (3), 1829.

147 Brew-Appiah, R.A.T., Rustgi, S., Claar, M., Langen, G., Kogel, K.H., and Weigel, D.V.W. (2010) Artificial microRNAs for silencing wheat proteins causing celiac disease. *Plant Biol*, Abs # P08017. http://abstracts.aspb.org/pb2010/public/P08/P08017.html

148 Choi, K., Park, C., Lee, J., Oh, M., Noh, B., and Lee, I. (2007) *Arabidopsis* homologs of components of the SWR1 complex regulate flowering and plant development. *Development*, **134** (10), 1931–1941.

149 Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M., and Weigel, D. (2005) Specific effects of microRNAs on the plant transcriptome. *Dev. Cell*, **8** (4), 517–527.

150 Lin, S.-S., Wu, H.-W., Elena, S.F., Chen, K.-C., Niu, Q.-W., Yeh, S.-D., Chen, C.-C., and Chua, N.-H. (2009) Molecular evolution of a viral non-coding sequence under the selective pressure of amiRNA-mediated silencing. *PLoS Pathog.*, **5** (2), e1000312.

151 Mathieu, J., Warthmann, N., Küttner, F., and Schmid, M. (2007) Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. *Curr. Biol.*, **17** (12), 1055–1060.

152 Kertesz, M., Iovino, N., Unnerstall, U., Gaul, U., and Segal, E. (2007) The role of site accessibility in microRNA target recognition. *Nat. Genet.*, **39** (10), 1278–1284.

153 de la Luz Gutierrez-Nava, M., Aukerman, M.J., Sakai, H., Tingey, S.V., and Williams, R.W. (2008) Artificial *trans*-acting siRNAs confer consistent and effective gene silencing. *Plant Physiol.*, **147** (2), 543.

154 Niemeier, S., Junior, L.A., and Merkle, T. (2010) Improvement of the design and generation of highly specific plant knockdown lines using primary synthetic microRNAs (pri-smiRNAs). *BMC Res. Notes*, **3** (1), 59.

155 Todesco, M., Rubio-somoza, I., Paz-ares, J., and Weigel, D. (2010) A collection of target mimics for comprehensive analysis of microRNA function in *Arabidopsis thaliana*. *PLoS Genet.*, **6** (7).

156 Kim, J. and Somers, D.E. (2010) Rapid assessment of gene function in the circadian clock using artificial microRNA in *Arabidopsis thaliana* mesophyll protoplasts. *Plant Physiol.*, **154** (2), 611–621.

157 Simón-Mateo, C. and García, J.A. (2006) MicroRNA-guided processing impairs Plum pox virus replication, but the virus readily evolves to escape this silencing mechanism. *J. Virol.*, **80** (5), 2429–2436.

158 Liu, D., Song, Y., Chen, Z., and Yu, D. (2009) Ectopic expression of miR396 suppresses GRF target gene expression and alters leaf growth in *Arabidopsis*. *Physiol. Plant*, **136** (2), 223–236.

159 Tagami, Y., Inaba, N., Kutsuna, N., Kurihara, Y., and Watanabe, Y. (2007) Specific enrichment of miRNAs in *Arabidopsis thaliana* infected with tobacco mosaic virus. *DNA Res.*, **14** (5), 227.

160 Gao, P., Bai, X., Yang, L., Lv, D., Pan, X., Li, Y., Cai, H., Ji, W., Chen, Q., and Zhu, Y. (2010) Osa-MIR393: a salinity- and alkaline stress-related microRNA gene. *Mol. Biol. Rep.*, **38**, 237–242.

26
Transcription Factors: Improving Abiotic Stress Tolerance in Plants

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Plant growth and productivity are greatly affected by environmental abiotic stresses, including drought, high salinity, high or low temperature, nutrient starvation, and excess metals in soils. After perceiving these stress signals, plants modulate the expression levels of various genes to adapt to and overcome environmental changes. Transcription factors thus play central roles in the regulatory networks that mediate the adaptation of plants to various environmental stresses. Although our knowledge of the transcription factors associated with abiotic stress response in crops is still limited, a number of such transcription factors have been recently identified, mainly in the model plant, *Arabidopsis thaliana*. In addition, several examples of transcription factors being successfully utilized to improve abiotic stress tolerance have now been reported, suggesting that this is a promising strategy to enhance stress tolerance in crops. In this chapter, we provide an overview of the present knowledge of plant transcription factors associated with various abiotic stress responses and their potential application to the enhancement of abiotic stress tolerance in plants.

26.1
Introduction

Plants must adequately adapt to fluctuations in the environment in which they grow as they cannot move from place to place. During the adaptation to stress conditions, plants modulate the expression of numerous genes. For instance, it has been shown by transcriptome analysis that drought stress induces expression of 277 genes, and represses another 79 genes, in *Arabidopsis* [1]. The expression levels of individual genes can be modulated through transcriptional control, alternative splicing events, and changes in RNA stability. Furthermore, the abundance of functional and active proteins can be regulated in response to stress signals by translational control, posttranslational modifications, and degradation mechanisms. Although abiotic

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stress responses in plants likely include various mechanisms to induce specific proteins, transcriptional control appears to be an important component of these processes.

Transcriptional control is typically exerted through the action of transcription factors that are specifically involved in particular signal responses. To more fully understand the molecular mechanisms underlying stress-responsive gene expression and thereby uncover potential strategies to improve stress tolerance in plants, the identification of the key transcription factors involved is absolutely necessary. Only a limited number of such analyses using crops have been conducted to date, but transcription factors associated with abiotic stresses, namely, drought, high salinity, cold and heat, nutrient starvation, and excess metals in the soil, have been identified in recent studies both in the model dicot *Arabidopsis* and in rice, a monocot plant and one of the most important crops in the world. Furthermore, several successes in improving stress tolerance have also been reported using these identified transcription factors. In this chapter, we provide an overview of these successful applications and also of the present knowledge of transcription factors involved in stress responses in plants.

26.2
Transcription Factors Involved in the Drought Stress Response

As water limitation severely impacts agricultural productivity, the enhanced tolerance to drought is one of the most important and sought after traits in the molecular breeding of crops. Drought stress exerts its effects on various cellular and molecular events in plants, particularly upon the expression of a variety of genes that are involved in not only stress tolerance but also in the enhancement of stress response pathways. Indeed, transcriptome analyses have now indicated that more than 300 transcripts are modulated by drought stress [1]. Owing to the diverse effects of drought stress, plant responses to water stress are thought to be regulated by an orchestrated but complex series of signaling networks, the details of which remain to be elucidated [2, 3]. Furthermore, as drought and salinity stresses have been found to cause similar changes in the transcriptome in *Arabidopsis* [4], the signaling pathways associated with these stresses may influence and activate each other or operate through a shared mechanism. In addition, a plant hormone, abscisic acid (ABA), has been shown to play a critical role in drought stress. ABA is dramatically produced under drought and salinity stress conditions and influences the expression of various genes that respond to them. Drought and salinity stress signaling and ABA signaling therefore are integrated into a complex regulatory network that forms part of the stress response mechanism in plants.

The cellular processes that operate in response to drought stress are initiated by the perception of these conditions by specific sensors. Recent studies have revealed the role of AtHK1/AHK1 in the perception of drought stress. AtHK1/AHK1 is a histidine kinase in the two-component signaling system, which also mediates

osmotic stress signaling in prokaryotes [5, 6]. Tran *et al*. have shown that AHK1 functions as an osmosensor and that its overexpression improves tolerance to drought stress in *Arabidopsis* [6]. RPK1, a receptor-like kinase that is localized to plasma membrane and controls several ABA responses in *Arabidopsis*, was also found to be involved in the early steps of osmotic stress signaling in plant cells [7, 8]. As the components that function directly downstream of AtHK1/AHK1 and RPK1 have yet to be identified, the molecular mechanisms underlying drought stress signaling in plants remain mostly unknown. However, in the past decade, the knowledge of the transcriptional control that functions during drought stress signaling has increased markedly through the identification of several transcription factors that play critical roles in the drought stress response in plants (Table 26.1).

26.2.1
DREB2 Transcription Factors

Similar to other types of transcriptional regulation, stress-responsive transcription is mediated by particular *cis*-acting elements and *trans*-acting transcription factors that recognize these *cis*-elements [3]. Indeed, the drought-responsive *cis*-element (DRE, 5′-TACCGACAT-3′) has been identified in the promoter regions of drought and salinity stress-inducible genes. Furthermore, a part of the DRE (5′-CCGAC-3′) has also been reported to function as the *cis*-element that regulates transcription from various cold-responsive gene promoters, and has been designated as the C-repeat (CRT) and the low-temperature-responsive element (LTRE) [9–12]. This suggests a close relationship between the regulatory mechanisms for drought and cold stress-responsive gene expression. Transcription factors that specifically recognize the DRE/CRT sequence have now been identified using yeast one-hybrid screening and are referred to as DREB1/CBF (DRE binding protein 1/CRT binding factor) and DREB2 [11, 13]. DREB1/CBF and DREB2 form the DREB/CBF family, a subfamily of the plant-specific AP2 (APETALA2)/ERF transcription factor family [14].

Although the function of DREB1/CBF was specifically characterized in the cold stress response in *Arabidopsis*, as discussed further later, expression of the *DREB2* genes, *DREB2A* and *DREB2B*, is induced by both dehydration and salinity stress but not by cold stress [13–15]. Hence, DREB2 appears to be involved in both the drought and the salinity stress response. This induction of *DREB2* genes precedes the induction of other stress-responsive genes that play roles in stress tolerance, such as *LEA* (late-embryogenesis abundant protein) genes, in agreement with the primary role of DREB2 in the drought stress response. However, the overexpression of *DREB2A* under the control of the cauliflower mosaic virus 35S RNA (35S) promoter does not affect the stress tolerance of transgenic *Arabidopsis* plants. This unexpected contradiction was resolved by further characterization of the DREB2A protein, which revealed that posttranslational modification is necessary for its full activation [13]. Indeed, Sakuma *et al*. showed that the negative regulatory domain exists in the central region of DREB2A and that deletion of this negative regulatory

Table 26.1 Transcription factors involved in drought, salinity, cold, and heat stress tolerance.

Class	Type	Gene	*cis*-elements	Induction	Stress	References
APETALA2 (AP2)	DREB/CBF	DREB1A/CBF3	DRE/CRT	Cold	Freezing, drought, salinity	[13, 45]
	DREB/CBF	DREB1B/CBF1	DRE/CRT	Cold	Freezing, drought	[44]
	DREB/CBF	DREB1C/CBF2	DRE/CRT	Cold	[freezing], [drought], [salinity]	[81]
	DREB/CBF	DREB1D/CBF4	DRE/CRT	Drought	Freezing, drought	[82]
	DREB/CBF	DREB2A	DRE/CRT	Drought, salinity	Drought, heat	[16, 18]
Leucine zipper (bZIP)	AREB	AREB1/ABF2	ABRE	Drought, salinity, ABA	Drought, salinity, glucose	[22–24]
	AREB	AREB2/ABF4	ABRE	Drought, salinity, ABA	Drought, salinity	[21]
	AREB	ABF3	ABRE	Drought, salinity, ABA	Drought, salinity	[21]
Nuclear transcription factor	NF-X	NF-X1	X1-box	Salinity, osmotic	Salinity, heat	[38, 80]
	NFYA	NFYA5	CCAAT	Drought, ABA	Drought	[39]
R2R3-MYB	MYB	AtMYB60	—	Downregulated by drought, ABA	[drought]	[41]
	MYB	AtMYB61	—	—	Decreased stomatal aperture	[42]
	MYB	AtMYB15	Myb recognition sequences	Cold	[freezing]	[62]
Homeodomain	OCP	OCP3	—	Downregulated by drought, ABA	[drought]	[43]
C_2H_2 zinc-finger	ZPT2	STZ	A(G/C)T	Cold, drought, salinity, ABA	Drought	[56]
Basic helix–loop–helix	MYC	ICE1	CATTTG	—	Freezing	[57, 58]
Calmodulin binding	CAMTA	CAMTA3	CM Motifs	—	—	[64]
Heat shock factor	HSF	AtHsfA2	HSE	Heat	Heat	[72]
	HSF	AtHsfA3	HSE	Heat	Heat	[78, 79]

"—" indicates that the binding sequence and the inducer have not been identified yet. Bracket indicates that gene disruption improved stress tolerance.

domain results in the production of a constitutively active form of this protein [16]. Hence, transgenic *Arabidopsis* plants overexpressing the constitutively active form of DREB2A showed a stronger tolerance to drought stress, accompanied by an upregulation of various stress-responsive genes [16]. The protein that interacts with the negative regulatory domain of DREB2A has recently been identified, as DRIP1 (DREB2A interacting protein 1), and is a C_3HC_4 RING domain-containing protein. Furthermore, negative regulatory mechanisms for DREB2A activity was suggested to involve 26S proteasome pathway-dependent proteolysis mediated by DRIP1 and its homologue DRIP2 [17].

Interestingly, the upregulated genes in the transgenic *Arabidopsis* overexpressing DREB2A include not only drought and salinity stress-responsive genes under these conditions but also genes encoding heat shock proteins (HSPs) [18]. As expected, transgenic *Arabidopsis* plants overexpressing such genes also show an increased tolerance to heat stress, whereas the corresponding knockout plants are impaired in this respect [18]. This observation also implies the presence of a complex regulatory network involving a crosstalk among various stress signaling pathways.

26.2.2
Transcription Factors that Interact with the ABA-Responsive Element in Drought Stress-Responsive Promoters

Drought and salinity stress conditions are partly mediated by ABA that induces expression of various genes through the ABA-responsive *cis*-element, the ABRE. Both DRE/CRT and ABRE have been found in many stress-responsive gene promoters, suggesting that ABRE also plays a role in stress-responsive transcription [3]. AREB/ABFs (ABRE binding proteins/ABRE binding factors) have been identified in *Arabidopsis*, and they are bZIP-type transcription factors [19, 20]. Consistent with their roles, the AREB/ABF transcripts accumulate in response to the exogenous application of ABA and to drought and salinity stress treatments [20]. Furthermore, the overexpression of ABF3 and ABF4/AREB2 in *Arabidopsis* increases the expression of ABA-responsive genes such as the LEA genes and results in the enhancement of ABA sensitivity, glucose sensitivity, and drought stress tolerance [21]. The overexpression of ABF2/AREB1 in *Arabidopsis* also enhances ABA and glucose sensitivity and improves drought tolerance. In contrast, the *abf2*/*areb1* mutants exhibited reduced sensitivity of glucose, which was not observed in the *abf3* and *abf4*/*areb2* mutants [22]. Similar effects caused by overexpression of ABF/AREB proteins but different phenotypes by disruption of *ABF*/*AREB* genes may be suggestive of redundant but different roles. Similar to DREB2A, the modification of the ABF2/AREB1 protein was found to be required for the full activation of ABF2/AREB1 [23, 24]. The overexpression of the active form of ABF2/AREB1 in *Arabidopsis*, therefore, increased ABA-responsive gene expression and drought tolerance more strongly [23, 24].

The transactivation activity of ABF2/AREB1 has been shown to depend on ABA and thus is lower in the ABA-insensitive mutant *abi1* [20]. Since the ABI1 protein is a phosphatase 2C (PP2C) [25, 26], ABA-dependent phosphorylation/dephosphorylation is thought to be involved in the activation of AREB/ABF proteins. Indeed,

Furihata *et al*. have shown that ABA treatment stimulates the activity of a 42-kDa protein kinase in *Arabidopsis* and induces the phosphorylation of Ser/Thr residues at R-X-X-S/T sites in AREB1 [24]. Furthermore, it has been revealed that the activity of the 42-kDa kinase toward ABF1, ABF2/AREB1, and ABI5 is impaired in the *snrk2.2snrk2.3* double mutant that harbors mutations in two genes encoding type-2 SNF1-related protein kinases (SnRK2.2/SRK2D and SnRK2.3/SRK2I) [27]. The *snrk2.2snrk2.3snrk2.6* triple mutant shows severe phenotypes in terms of the ABA sensitivity, the phosphorylation of ABF/AREBs, and the ABA-dependent gene expression [28–31]. The SnRKs have, therefore, been shown to be involved in the activation of AREB/ABFs. It was recently revealed that ABA promotes interactions between ABA receptors, PYR/PYL/RCARs, and PP2Cs, ABI1 and ABI2, which are negative regulators of ABA signaling [32, 33], and thereby inhibits PP2C activity to control ABA signaling [34, 35]. Therefore, ABA likely regulates ABF/AREB activity through the PYR/PYL/RCAR–PP2C–SnRK cascade and then contributes to the drought and salinity stress response pathways, although the exact mechanism is unknown.

26.2.3
Additional Transcription Factors Involved in the Drought Stress Response

Transcription factors of the MYB, NAC, and other families have also been suggested to play roles in the drought response in plants [3]. One of these is a novel C_2H_2-type transcription factor, DST (DROUGHT AND SALT TOLERANCE), which controls the expression of genes involved in H_2O_2 homeostasis and mediates H_2O_2-induced stomatal closure and abiotic stress tolerance in rice [36]. Both H_2O_2 and hydroxyl radicals are typical reactive oxygen species (ROS). ROS production is induced by both abiotic and biotic stress, including high light, osmotic stress, and pathogen attack, and ROS detoxification is one of the most important steps in stress tolerance. Further characterization of DST might, therefore, clarify the interaction between ROS and ABA signaling pathways in the drought and salinity stress responses.

Another transcription factor associated with drought and salt stresses is an *Arabidopsis* factor that is structurally related to the human NF-X1 protein (nuclear transcription factor X-box binding 1) and contributes to salt and defense responses [37, 38]. Li *et al*. have shown that NFYA5, a drought stress-inducible nuclear transcription factor Y, plays a role in controlling stomatal aperture and drought tolerance [39]. Several transcription factors have also been found to regulate stomatal apertures under conditions of drought stress. Overexpression of an NAC transcription factor in rice, SNAC1 (stress-responsive NAC 1), which is expressed in guard cells, leads to an increased ABA sensitivity and stomatal closure and results in improved drought and salt tolerance [40]. Two MYB transcription factors, AtMYB60 and AtMYB61, which are also expressed mainly in guard cells, have been functionally characterized as important modulators of stomatal aperture and drought tolerance in *Arabidopsis*. AtMYB60 is a negative regulator of stomatal closure under drought stress conditions and the *atmyb60* null mutation results in the constitutive reduction of stomatal opening, whereas AtMYB61 is a positive regulator of this process and its loss

of function results in more-open stomata [41, 42]. *OCP3*, encoding a transcription factor of the homeodomain family, also plays a role in controlling the ABA-induced stomatal aperture and drought resistance [43]. These factors are likely to be associated with complex mechanisms underlying drought stress signaling in stomata.

26.3 Transcription Factors that Mediate the Response to Cold Stress

As the expression of DREB1/CBF proteins (DREB1A-C/CBF1-3) is induced by cold stress [13, 15], these factors appear to be specifically engaged in the transcriptional regulation of cold stress-responsive genes. Consistent with their induction, the overexpression of *DREB1B/CBF1* under the control of the 35S promoter increases tolerance to cold stress in *Arabidopsis* [44]. Interestingly, however, the overexpression of *DREB1/CBF* enhances the tolerance to not only cold stress but also drought and salinity stress conditions [13, 45, 46], suggesting either that a crosstalk exists among stress signaling pathways or that a mechanism shared by different stress signaling pathways is activated. Although the improved tolerance to cold stress indicates that the utilization of *DREB1/CBF* is an effective approach to improve cold stress tolerance, transgenic *Arabidopsis* lines overexpressing *DREB1/CBF* also show severe growth defects [45]. Hence, Kasuga *et al*. were able to overexpress *DREB1/CBF* under stress conditions using only a stress-inducible promoter [45]. As the newly generated transgenic *Arabidopsis* did not manifest any negative effect in terms of plant growth [45], the strategy with *DREB1/CBF* appears to be now a practical approach that can be applied to crop improvement. In fact, the overexpression of *DREB1/CBF* genes also increased tolerance to freezing, chilling, and drought stress in various plant species, including *Brassica napus*, tobacco, tomato, rice, wheat, and maize [47–54]. The overexpression of *DREB1/CBF* in *Arabidopsis* triggers the upregulation of not only various stress-responsive genes including LEA protein and cold-inducible KIN protein genes but also a C_2H_2 zinc finger transcription factor gene. This gene, termed *STZ*, is one of the direct target genes of DREB1/CBF [55, 56]. Because the overexpression of STZ, which functions as a transcriptional repressor, also enhances the tolerance to drought stress [56], the transcriptional cascade including DREB/CBF and STZ appears to play a key role in the cold stress response in *Arabidopsis*.

The presence of a complex regulatory mechanism underlying the expression of DREB1/CBF in response to stress signals has been revealed. The *ICE1* (*inducer of CBF expression 1*), *HOS1* (*high expression of osmotically responsive gene 1*), and *MYB15* genes were found to encode factors involved in this regulatory mechanism (Figure 26.1). ICE1, a basic helix–loop–helix (bHLH) transcription factor, is a major positive regulator of *CBF3* through the binding of multiple *cis*-regulatory elements in the promoter and promotion of transcription [57, 58]. On the other hand, HOS1 is a RING-type ubiquitin E3 ligase that negatively regulates *DREB1/CBF* gene expression [59]. Miura *et al*. showed that the low-temperature-induced sumoylation of ICE1 is mediated by the SIZ1 protein, a SUMO E3 ligase, and that this process is a key regulatory component of cold stress-responsive gene expression [60]. This process is

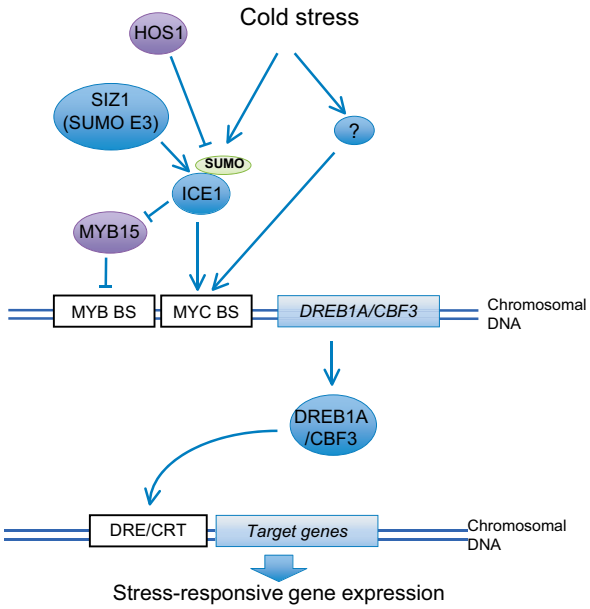

Figure 26.1 The transcriptional network that operates during the cold stress response. The DREB1A/CBF3 transcription factor that binds the DRE/CRT *cis*-acting elements and regulates transcription is expressed under the control of multiple components, including MYB15, ICE1, SIZ1, and HOS1, in response to cold stress.

inhibited by HOS1, which mediates the ubiquitination and degradation of ICE1 [58, 61]. MYB15 that interacts with ICE1 is another regulator that binds and negatively regulates transcription from the *CBF* promoters [62]. Thus, cold stress influences *DREB1/CBF* gene expression via protein sumoylation and ubiquitination and fine-tunes the expression of various stress-responsive genes during the stress tolerance response in plants (Figure 26.1) [63].

On the other hand, CAMTA3, a member of the CAMTA (calmodulin binding transcription activator) family of transcription factors, has been shown to be a positive regulator of the *DREB1C/CBF2* gene [64]. The *camta3* mutant plants show a reduced induction of *CBF2* under cold stress conditions, and the *camta1 camta3* double mutant shows a reduced freezing tolerance. This suggests a connection between calcium signaling and cold-regulated gene expression.

26.4
Transcription Factors Mediating the Response to Heat Stress

Heat stress tolerance (thermotolerance) is also under the control of coordinated signaling pathways [65]. Heat stress or heat shock induces the synthesis and accumulation of heat shock proteins in both plants and animals. The HSPs comprise

five protein families, namely, the HSP100, HSP90, HSP70, and HSP60 families and the small HSPs (sHSPs) [66]. HSPs primarily function as molecular chaperones to prevent the aggregation of denatured proteins caused by heat shock and to promote the appropriate refolding of denatured proteins [67].

The heat-inducible expression of *HSP* genes is regulated by heat shock transcription factors (HSFs) that are conserved in eukaryotes. HSFs bind to heat shock-responsive elements (HSEs), which are conserved *cis*-elements in the *HSP* gene promoters [68]. Among 21 HSFs in *Arabidopsis*, AtHsfA1a and AtHsfA1b have already been shown to play important roles in the expression of *HSP* genes in the early phase of the heat shock response [69, 70]. Furthermore, Guo *et al.* have determined the AtHsfA1a binding sites *in vivo* and shown that they are located in the promoter regions of a set of classical heat shock protein genes and a transmembrane CLPTM1 family protein gene [71]. A heat stress-induced HSF, AtHsfA2, has also been shown to activate *HSP* expression and then enhance acquired thermotolerance in *Arabidopsis* [72].

The heat stress response is mediated through the modulation of HSF activity by HSPs. Under nonstress conditions, constitutively expressed HSFs are inactivated by the binding of HSPs and maintained in the cytosol. However, when plants perceive heat stress, the HSP–HSF complexes dissociate and the HSFs localize to the nucleus to regulate gene expression [73–76]. Furthermore, the activity of HSFs is also known to be regulated by additional mechanisms. For instance, the activity of HSFs is modulated through phosphorylation/dephosphorylation events in response to heat stress [74, 77]. On the other hand, the heat stress response has also been found to be decreased by the heat shock factor binding protein 1 (HSBP1) that binds HSFs and then inhibits HSF activity [74]. Interaction with HSBP1 thus represents another regulatory mechanism for HSF activity.

Interestingly, the drought stress-responsive transcription factor, DREB2A, also influences heat tolerance [16, 17]. The overexpression of *DREB2A* in *Arabidopsis* increases heat tolerance by inducing the expression of the *AtHsfA3* gene [78, 79]. NF-X1 that was initially identified as a transcription factor associated with the salinity stress response [37, 38] has also been reported to contribute to heat tolerance [80]. These findings consistently indicate a connection between heat stress and other stress signaling pathways in the complex regulatory network that mediates stress responses in plants.

26.5
Transcription Factors Involved in Nutrient Deficiency

Plant nutrients in the soil, particularly nitrogen and phosphorus, are major factors that affect plant growth. Plants have, therefore, developed mechanisms to effectively absorb and utilize plant nutrients. In the past decade, several transcription factors involved in nutrient deficiency have been identified. Furthermore, genetic modifications based on these factors suggest that they may be potent tools for improving plant growth under nutrient-deficient conditions (Table 26.2).

Table 26.2 Transcription factors involved in nutrient responses.

Nutrient	Gene	Gene family	Species	References
N	*ANR1*	MADS	*A. thaliana*	[102, 103]
	NSR1	MYB	*A. thaliana*	[95]
	NLP7	RWP-RK	*A. thaliana*	[96]
	LBD37/38/39	LBD	*A. thaliana*	[97]
	GNC	GATA	*A. thaliana*	[99]
	Dof1	Dof	*Zea mays*	[101, 102]
P	*PHR1*	MYB	*A. thaliana*	[94, 105]
	PHL1	MYB	*A. thaliana*	[105]
	PHR2	MYB	*A. thaliana*	[95]
	OsPHR1	MYB	*O. sativa*	[108]
	OsPHR2	MYB	*O. sativa*	[108]
	MYB62	MYB	*A. thaliana*	[115]
	ZAT6	C_2H_2 zinc finger	*A. thaliana*	[116]
	WRKY75	WRKY	*A. thaliana*	[117]
	WRKY6	WRKY	*A. thaliana*	[118]
	BHLH32	bHLH	*A. thaliana*	[120]
	OsPTF1	bHLH	*O. sativa*	[121]
S	*SLIM1*	EIL	*A. thaliana*	[124]
Fe	*FER*	bHLH	*Lycopersicon esculentum*	[130]
	FIT1	bHLH	*A. thaliana*	[132, 133]
	AtbHLH38/39	bHLH	*A. thaliana*	[134, 135]
	OsIRO2	bHLH	*O. sativa*	[136]
	IDEF1	ABI3/VP1	*O. sativa*	[139]
	IDEF2	NAC	*O. sativa*	[143]
Zn	*bZIP19/23*	bZIP	*A. thaliana*	[149]

26.5.1 Transcription Factors Involved in the Nitrogen Response

Nitrogen is a macronutrient that is required in abundance for plants. Plants actively attempt to obtain inorganic nitrogen in the soil due to the constitutive constraints on nitrogen availability in natural ecosystems. Although plants can use both nitrate and ammonia in the soil as a nitrogen source, nitrate is the major source for land plants. The possible roles of nitrate, nitrite, and glutamine as nitrogen signals that regulate cellular processes in plant cells have been proposed in the past [83–87], and nitrate has been well characterized as the main nitrogen signal. Therefore, both nitrate-inducible responses and nitrogen-deficient responses are likely to be closely related to nutrient-deficient stress responses. Transcription factors involved in nitrate-inducible and nitrogen-deficient responses are given equal weight in this section.

Transcriptome analysis of nitrogen-deficient response in rice roots has revealed that the modulation of a number of genes, including putative transcription factor genes, is induced by nitrogen-deficient conditions [88]. However, no additional information on these genes has yet been reported. On the other hand, the nitrate-inducible response in plants has been analyzed in more detail. Transcriptome analysis has revealed that the expression of numerous genes is modulated in response to the nitrate supply even in the *Arabidopsis* mutant that has little nitrate reductase (NR) activity [84, 89, 90]. Thus, nitrate has been suggested to function as a signal molecule and to directly modulate gene expression because it is reduced to ammonium by reductive reactions catalyzed by two enzymes, NR and nitrite reductase (NIR), and then assimilated into glutamine. Furthermore, a *cis*-element that is sufficient to drive nitrate induction was recently identified in promoter analysis of the *Arabidopsis* gene for NIR [91]. This element did not respond to glutamine, although the expression of *NIR* was found to be repressed by glutamine, in an experiment that defined the multiple *cis*-elements involved in the nitrogen response [91].

Transcription factors that bind directly to the identified nitrate-responsive *cis*-element have not been elucidated as yet. However, several genes encoding transcription factors are known to respond to nitrogen starvation or nitrate supply. *Arabidopsis ANR1*, which encodes an MADS transcription factor, is expressed preferentially in roots [92]. Although lateral roots proliferate in response to a localized nitrate supply, this proliferation does not occur when expression of *ANR1* is suppressed or *ANR1* is disrupted [92, 93]. *ANR1* was also found to be induced by nitrate starvation and repressed by the subsequent nitrate resupply, suggesting a possible feedback regulation of the lateral root growth rates via the nitrogen status of the plant [93]. There are also seven other MADS-box genes, which are slightly upregulated by nitrate starvation [93].

An *Arabidopsis* gene for NSR1, which is structurally close to the PHR1 (PHOSPHORUS STARVATION RESPONSE 1) transcription factor involved in the phosphate (Pi) starvation response [94], is also induced by nitrate starvation, although it has not yet been characterized in any detail [95]. The *Arabidopsis NLP7* gene that encodes a RWP-RK transcription factor also appears to be involved in the nitrogen response, although its expression is not regulated by the nitrogen source or by the presence of nitrate [96]. When the *NLP7* gene was disrupted in *Arabidopsis*, the plants exhibited the features of nitrogen-starved plants, such as an increase in the ratio of root/shoot, a longer primary root, and a higher lateral root density. Induction of the nitrate transporter genes (*NRT2.1* and *NRT2.2*) and the NR genes (*NIA1* and *NIA2*) by nitrate was found to be impaired in the *nlp7* mutant. Thus, NLP7 is a putative regulatory protein for nitrogen assimilation, although there is no direct evidence that NLP7 directly regulates the expression of nitrate-inducible genes.

The nitrate-inducible *LBD37/38/39* genes of *Arabidopsis* are members of the *LBD* (*LATERAL ORGAN BOUNDARY DOMAIN*) family of transcription factors [97]. Because these genes are induced by not only nitrate but also other

nitrogen sources, ammonium and glutamine, these genes are nitrogen responsive rather than nitrate responsive. Overexpression of *LBD37*, *LBD38*, or *LBD39* suppresses the expression of *PAP1* and *PAP2* that encode MYB transcription factors regulating expression of genes involved in anthocyanin synthesis [98]. The overexpression of *LBD37*, *LBD38*, or *LBD39* also repressed the expressions of nitrate transporter genes and NR genes [97], suggesting that LBD37/38/39 proteins play negative roles in the nitrogen response.

An *Arabidopsis* gene, *GNC* (*GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED*), was also shown to be a nitrate-inducible gene [99]. This gene encodes a GATA transcription factor and is expressed in leaves and buds in response to nitrate, but not in roots. In the *gnc* mutant, the chlorophyll level is reduced and the expression of genes involved in carbon metabolism is repressed. The *gnc* mutant was also found to be more sensitive to exogenous glucose, whereas transgenic *Arabidopsis* overexpressing *GNC* is less so. Carbon and nitrogen metabolism are closely linked [100] because nitrogen assimilation requires not only inorganic nitrogen but also the carbon skeleton 2-oxoglutarate (2-OG) that is produced from photoassimilated carbohydrates. On the basis of the phenotype of the *gnc* mutant and the *GNC* overexpressors, GNC is proposed to function in the regulation of carbon and nitrogen metabolism in response to nitrate.

Although recent studies have identified several transcription factors involved in the nitrate response and/or nitrate status in plant cells, the enhancement of nitrogen assimilation, one of the most important agricultural traits sought after by breeders, has not been achieved yet using these factors. However, such enhancement was achieved using Dof1, a member of plant-specific Dof transcription factor family (Figure 26.2) [101]. The transgenic *Arabidopsis* lines expressing the Dof1 transcription factor, a putative regulator of 2-OG production, were found to show enhanced nitrogen assimilation and a larger pool of organic nitrogen and thus show better growth under the low-nitrogen conditions (Figure 26.2) [102]. This suggested the importance of the coordinated modulation of carbon and nitrogen metabolism in improving the adaptive ability of plants to nitrogen-deficient environments.

26.5.2
Phosphate Starvation-Responsive Transcription Factors

26.5.2.1 PHR1 Involved in Phosphate Starvation Response

Phosphorus is a major structural component of nucleic acids and membrane lipids and takes part in the regulation of many biochemical and physiological processes. Plants acquire phosphorus as inorganic phosphate (Pi). Several transcription factors involved in the Pi starvation response have been recently identified in vascular plants. Among these, PHR1 from *Arabidopsis thaliana* was the first to be isolated and is thus the most well characterized of these transcription factors [94]. The gene encoding PHR1 was identified by a genetic screen of a transgenic *Arabidopsis* line harboring the *AtIPS1::GUS* reporter construct that was specifically responsive to Pi starvation. In the *phr1* mutant, the root/shoot ratio was impaired and anthocyanin did not

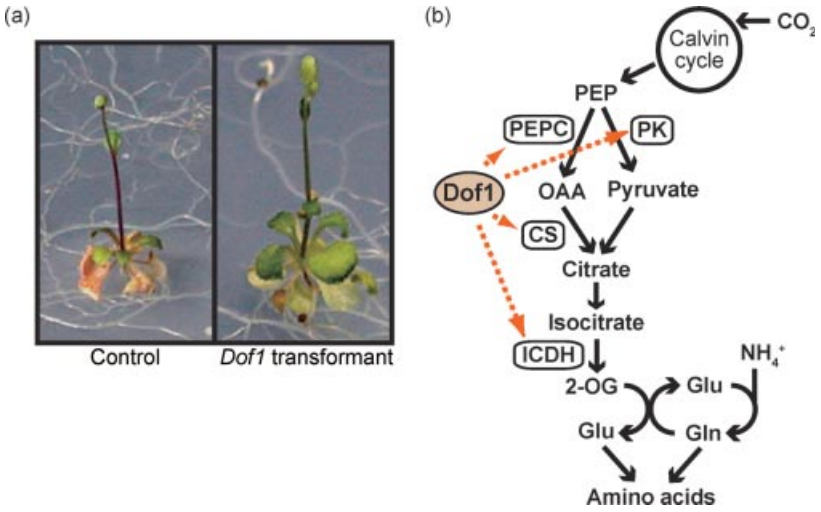

Figure 26.2 Improvement of plant growth by Dof1 under the low-nitrogen conditions. (a) The glutamine content is lower in control *Arabidopsis* plants than the transgenic *Arabidopsis* plants expressing the Dof1 transcription factor, and control *Arabidopsis* plants withered their leaves earlier when they were grown under the low-nitrogen conditions. (b) The metabolic pathway for nitrogen assimilation in plants. PEP: phosphoenolpyruvate; OAA: oxaloacetate; PEPC: phosphoenolpyruvate carboxylase; PK: pyruvate kinase; CS: citrate synthase; ICDH: isocitrate dehydrogenase. PEPC, PK, CS, and ICDH genes are putative target genes of the Dof1 transcription factor.

accumulate under Pi starvation conditions. Furthermore, the cellular Pi content was lower in the *phr1* mutant than in the wild-type *Arabidopsis* under Pi-sufficient conditions. In accordance with the *phr1* mutant phenotype, the expression of several Pi-responsive genes including genes encoding Pi transporters, acid phosphatases, and enzymes involved in anthocyanin biosynthesis was found to be impaired in the *phr1* mutant [94, 103, 104]. Conversely, when *PHR1* was overexpressed in *Arabidopsis*, the Pi content in the shoots increased, accompanied by the elevated expression of several Pi starvation-responsive genes [104].

PHR1 is a member of the MYB-CC gene family, which is a subtype of the MYB superfamily and includes 15 members in *Arabidopsis* [105]. Among these, PHL1 (PHR1-LIKE1) is phylogenetically most closely related to PHR1. It has been shown that PHR1 and PHL1 are functionally redundant in *Arabidopsis* [105] and form heterodimers to bind an imperfect palindromic DNA sequence called P1BS (5′-GNATATNC-3′), which are enriched in the promoter regions of many Pi starvation-responsive genes [94, 105–107]. Although *PHR1* expression is not affected by the Pi status, another *PHR1* homologue, *PHR2*, was found to be induced by Pi deprivation [94, 95]. Thus, PHR2 could also be involved in the Pi starvation response, although it has not yet been characterized in sufficient detail. Although rice has two *PHR1* homologues, *OsPHR1* and *2*, only the *OsPHR2* overexpressor accumulates

excess Pi in its shoots with upregulated levels of Pi starvation-responsive gene transcripts, similar to *PHR1* overexpressing *Arabidopsis* [104, 108].

In addition to the analyses of the physiological roles of PHR1 and related factors, the molecular regulation of PHR1 expression has also been studied. Because the expression levels of *PHR1* and the nuclear localization of the PHR1 protein are unchanged by the Pi status, posttranslational modifications can be considered to be the regulatory mechanism for PHR1 activity [94]. Indeed, the PHR1 protein was revealed to be sumoylated by SIZ1, a small ubiquitin-like modifier (SUMO) E3 ligase [109]. The functional relationship between PHR1 activity and sumoylation was further shown by the modified expression of target genes of PHR1 in the *siz1* mutant. These target genes, *AtIPS1* and *AtRNS1*, were induced more slowly in the *siz1* mutant than the wild type by Pi starvation, in agreement with the putative positive regulation of PHR1 by SIZ1. The *siz1* mutant also exhibited more severe Pi starvation phenotypes under such conditions, such as retarded primary roots, extensive lateral roots, and root hair development, an increase in the root/shoot growth ratio, and anthocyanin accumulation, although the intracellular Pi content in the *siz1* mutant was similar to that of wild- type *Arabidopsis* [109].

A microRNA, miR399, was found to be another of the PHR1 targets [103]. In plants, miRNAs recognize specific mRNA sequences based on sequence complementarity and function to cleave their target mRNAs. As the target of miR399 is the mRNA of *PHO2* encoding a ubiquitin-conjugating E2 enzyme, *Arabidopsis* lines overexpressing miR399 exhibit a phenotype similar to that of the *pho2* mutant [103, 110]. Interestingly, other small noncoding RNAs from *AtIPS1* and *At4* contain a sequence motif that is partially complementary to miR399 and inhibit the cleavage of *PHO2* mRNA by miR399 [111, 112]. As the expression of *AtIPS1/At4* is also induced by Pi starvation, differential induction modes of miR399 and *AtIPS1/At4* and/or the translocation of miRNA399 has been suggested to regulate the *PHO2* mRNA level in response to changes in the availability of Pi [113].

26.5.2.2 **Additional Transcription Factors Induced by Phosphate Starvation**

In addition to PHR1 and its homologues, several other transcription factors have been shown to be involved in the Pi starvation response. Some of them were identified through microarray analysis of Pi starvation-responsive genes [106, 114]. *MYB62* of *Arabidopsis* encodes an R2R3-type MYB transcription factor and its expression in leaves is induced by Pi starvation [115]. When *MYB62* is overexpressed, the root system architecture is altered. Furthermore, the root/shoot ratio increases under conditions of Pi sufficiency, anthocyanin accumulates, and the acid phosphatase activity is elevated. The expression of several Pi starvation-induced genes has also been shown to be suppressed in *MYB62* overexpressing lines. Thus, MYB62 is suggested to function as a repressor of the Pi starvation response [115].

Arabidopsis ZAT6, encoding a C_2H_2 zinc finger transcription factor, was also found to be induced by Pi starvation [116]. Overexpression of *ZAT6* in *Arabidopsis* leads to an

increased accumulation of anthocyanin and acid phosphatase secretion, a reduction both in the Pi uptake and in the total Pi content, and retardation of primary roots in young seedlings. However, when plants overexpressing *ZAT6* become older, they form longer lateral roots, which leads to an increased root/shoot ratio and total Pi content. Moreover, the expression of several Pi starvation-inducible genes is suppressed in the *ZAT6* overexpressing lines, indicating that *ZAT6* is also a repressor of the Pi starvation response.

WRKY75 is another Pi starvation-inducible gene [117]. The suppression of *WRKY75* by RNAi results in an increased anthocyanin accumulation, reduced Pi uptake, and reduced acid phosphatase activity. In addition, the lateral root length and number and the root hair number are increased in these knockdown plants, leading to an increased Pi content. Furthermore, the expression of Pi starvation-inducible genes is also suppressed in the *WRKY75* RNAi lines. Hence, *WRKY75* has been suggested to be a positive regulator of Pi starvation response. The involvement of another WRKY gene, *WRKY6*, in the Pi starvation response was also indicated by the phenotype of *Arabidopsis* lines overexpressing this gene under low Pi conditions. Similar to the *pho1* mutant, these transgenic lines were found to be defective in loading Pi into the xylem [118, 119]. It was shown that WRKY6 can bind to two W-boxes of the *PHO1* promoter to repress the *PHO1* gene. In addition, WRKY42, the closest homologue of WRKY6, also represses *PHO1* expression. Furthermore, degradation of the WRKY6 protein by the 26S proteasome under low Pi conditions was found to cause the repression of *PHO1* [118], indicating a molecular mechanism for Pi starvation that is mediated by a transcription factor.

Another Pi starvation-inducible gene in *Arabidopsis*, *BHLH32*, encodes a bHLH transcription factor [120]. In the *bhlh32* mutant grown under Pi-sufficient conditions, the Pi starvation-inducible expression of PPCK (phosphoenolpyruvate carboxylase kinase) genes, anthocyanin accumulation, and root hair formations were promoted. Also, the Pi content in the mutant was higher than that of the wild-type *Arabidopsis*. Thus, BHLH32 is likely to be a negative regulator of the Pi starvation response.

The *OsPTF1* (*Rice Pi starvation-induced transcription factor 1*) gene encoding a bHLH transcription factor was identified using a subtractive hybridization method with a cDNA library that was constructed using mRNA from rice roots subjected to Pi starvation [121]. The expression of *OsPTF1* was found to be upregulated by Pi starvation in roots but remained constitutively active in shoots. Transgenic rice plants overexpressing *OsPTF1* showed increase in the tiller number, shoot and root biomass, and the Pi content under low Pi conditions compared to wild-type rice plants.

Although the involvement of several Pi starvation-inducible transcription factor genes in the Pi starvation response has been conclusively shown by phenotypic analyses of the corresponding mutants and/or transgenic plants, the molecular functions of these factors and their target genes remain to be clarified.

26.5.3
Transcription Factors Associated with the Sulfur Starvation Response

Sulfur is an essential macronutrient required for plant growth. Plants uptake sulfate in the soil and use it for synthesis of cysteine and methionine, which are further utilized for the biosynthesis of other sulfur-containing organic compounds. Because of the important roles a number of sulfur-containing compounds play in plants, sulfur starvation induces the expression of sulfate transporters and activates the uptake of sulfate in roots. It is known that SULTR1;1 and SULTR1;2 are the high-affinity sulfate transporters in *Arabidopsis*, the transcripts of which are upregulated by sulfur starvation and downregulated by the sulfur-containing metabolites, cysteine and glutathione [122]. Analysis of the *SULTR1;1* gene promoter revealed the presence of a sulfur-responsive *cis*-element (SURE), which directs both the induction of this gene by sulfur starvation and its repression by cysteine and glutathione [123]. Furthermore, a 7-bp sequence within the SURE was found to be the core sequence that functions in the response to sulfur starvation. This core sequence is present in many sulfur starvation-responsive gene promoters [123], although not in the *SULTR1;2* promoter. No SURE binding protein has yet been identified.

Although transcription factors directly regulate the expression of sulfur starvation-responsive genes, a mutation in a gene encoding the SLIM1 (SULFUR LIMITATION 1) transcription factor has been found to affect the sulfur starvation-responsive gene. *SLIM1* was identified through the analysis of an *Arabidopsis* mutant in which the expression of GFP originating from the *SULTR1;2* promoter::GFP construct was abolished under sulfate starvation conditions [124]. SLIM1 is a member of the EIN3/EIL family that includes the ethylene-responsive transcription factors, EIN3 and EIL1, in *Arabidopsis* [125], but appears to be functionally distinguishable from other members of the EIL family, as they cannot rescue the phenotype of the *slim1* mutants [124]. In the *slim* mutant, the sulfate uptake activity was reduced and the growth of primary roots was inhibited under the sulfur-limiting conditions. Transcriptome analysis of the *slim1* mutant revealed that *SLIM1* functions to upregulate the genes for several isoforms of sulfate transporters, including SULTR1;1 and SULTR1;2 that function to uptake sulfate and SULTR4;2 that functions to release sulfate from the vacuoles in root tissues [122, 126] in response to sulfur starvation. In addition, a serine acetyltransferase gene, *SERAT3;1*, involved in cysteine synthesis, and a thioglucosidase gene involved in the degradation of glucosinolates for catabolic sulfur recycling were found to be upregulated under sulfate starvation conditions in the SLIM1-dependent manner [127]. The expression of genes encoding enzymes involved in the biosynthesis of glucosinolate, a major sulfur-containing secondary metabolite [128], was also affected by the *slim1* mutation. Hence, SLIM1 is proposed to be a global regulator of sulfate metabolic pathways [124]. Since the level of *SLIM1* mRNA is not modulated by changes in the sulfur conditions, posttranscriptional modifications in response to sulfur starvation may be critical for controlling SLIM activity [124]. Despite the close relationship between the SLIM activity and the sulfur starvation response, it is still unknown whether SLIM directly regulates genes that function in the regulatory network underlying the sulfur starvation response.

26.5.4
Iron Response-Related Transcription Factors

Iron is an essential micronutrient for plants and is required for cellular processes including photosynthesis and nitrogen fixation in legumes. Under aerobic or alkaline conditions, iron is present in an oxidized low-soluble Fe(III) form in the soil, which is not readily available for plants. Plants thus need to develop a mechanism for the effective acquisition of iron in the soil. Higher plants, with the exception of grasses, release protons around the roots to lower the pH, induce the expression of Fe(III) chelate reductase to reduce iron to the more-soluble Fe(II) form, and induce an Fe(II) transporter system for the uptake of Fe(II) into the root epidermis. This plant response to iron deficiency is called the strategy I response [129]. On the other hand, grasses have evolved a distinct strategy for iron uptake that is known as strategy II. They produce molecules of the mugineic acid family called phytosiderophores (PSs). PSs are secreted around the roots to form the soluble Fe(III)–PS complex, which is then taken up into the root cells through the Fe(III)–PS complex transporters [129].

The tomato FER was the first identified transcription factor associated with the strategy 1 response [130]. FER is a bHLH transcription factor expressed in roots under iron-deficient conditions [130]. Its abundance appears to be posttranscriptionally modulated, as it is detectable only when the iron levels are low. This is despite the fact that *FER* is constitutively and strongly expressed under the control of the 35S promoter [131]. In the *fer* mutant tomato, there is a decrease in Fe(III) chelate reductase activity and expression of the Fe(II) transporter gene [130]. Similarly, *Arabidopsis* FIT1/FRU (Fe-DEFICIENCY-INDUCED TRANSCRIPTION FACTOR1/FER-LIKE REGULATOR OF IRON UPTAKE), an orthologue of tomato FER [130, 131], was shown to be involved in the expression of the Fe(III) chelate reductase (FRO2) gene and promote the accumulation of Fe(II) transporter (IRT1) in the roots under iron-deficient conditions [132, 133]. The overexpression of FIT1 did not affect the expression of *FRO2* and *IRT1*. However, when FIT1 was coexpressed strongly with AtbHLH38 or AtbHLH39, which physically interact with FIT1, both *FRO2* and *IRT1* were constitutively expressed regardless of the iron conditions [134]. Furthermore, Fe(III) chelate reductase activity was higher in the transgenic *Arabidopsis* lines, coexpressing FIT1 and AtbHLH38 or AtbHLH39, than in the wild-type and transgenic lines overexpressing *FIT1*, *AtbHLH38*, or *AtbHLH39* alone. Moreover, the coexpressors accumulate IRT1 protein regardless of the iron conditions, resulting in the accumulation of more iron in their shoots [134]. As the *AtbHLH38* and *AtbHLH39* genes are upregulated under conditions of iron deficiency [134, 135], the cooperative action of FIT1, and AtbHLH38 and AtbHLH39, has been shown to play a critical role in the response to this stress.

The transcription factor involved in the strategy II response has also been identified through the profiling of iron deficiency-induced genes [136, 137]. This was a rice bHLH transcription factor, OsIRO2. Under iron-deficient conditions, rice plants overexpressing *OsIRO2* exhibited improved growth, whereas the knockdown lines generated by RNAi showed a reduced biomass and accumulated less iron [137]. The expression of many genes involved in PS biosynthesis and a Fe(III)-PS transporter

gene, *OsYSL15*, was enhanced in the *OsIRO2* overexpressors and repressed in the RNAi lines [137, 138]. Recently, it has been shown that *OsIRO2* expression is regulated by another transcription factor, IDEF1 [139]. IDEF1 is a member of the ABI3/VP1 family of transcription factors and binds to the iron deficiency-responsive *cis*-element, IDE1, which is present in the promoter of the barley *IDS2* gene involved in PS biosynthesis [139, 140]. Transgenic rice expressing *IDEF1* under the control of an iron deficiency-inducible promoter exhibited improved tolerance to iron deficiency [139], accompanied by a stronger expression of genes involved in PS biosynthesis, including *OsYSL15*, a Fe(II) transporter gene *OsIRT1*, and a metal-nicotianamine transporter gene *OsYSL2* [139, 141, 142].

Another iron deficiency-responsive *cis*-element, IDE2, was found in the promoter of the barley *IDS2* gene [140], and it has been shown that a rice NAC transcription factor, IDEF2, binds to this *cis*-element [143]. When IDEF2 function was repressed using the RNAi technique and chimera repressor gene-silencing technology (CRES-T), the resulting rice plants accumulated more iron in both their shoots and their roots under iron-sufficient conditions. On the other hand, under conditions of iron deficiency, the iron concentration appears to be lower in the shoots of the RNAi and CRES-T rice lines, whereas the iron concentrations in the roots were higher in transgenic lines. This phenomenon was explained by the hypothesis that the severe suppression of *OsYSL2* in the *IDEF2 RNAi* rice and the CRES-T rice lines under iron deficiency might prevent OsYSL2 from translocating iron from the roots to the shoots [142, 143]. Transcripts of *IDEF1* and *IDEF2* are constitutively expressed regardless of the iron conditions, and IDEF proteins may therefore be modified and regulated posttranscriptionally [139, 143].

26.5.5
Zinc Deficiency-Responsive Transcription Factors

Zinc is an essential micronutrient and an essential cofactor for many transcription factors, protein interaction domains, and enzymes both in plants and in animals [144]. Members of the ZIP family of metal transporters play a major role in zinc uptake in plants [145]. In *Arabidopsis*, a *ZIP* transporter gene, *ZIP4*, is strongly induced in response to zinc deficiency [146]. Using *ZIP4* promoter fragments as baits in a yeast one-hybrid assay, two homologous transcription factors, bZIP19 and bZIP23, were identified recently [147]. These factors act redundantly, and the expression levels of both genes are higher under zinc-deficient conditions. Furthermore, the *bzip19 bzip23* double mutant exhibited a zinc deficiency-hypersensitive phenotype under zinc-deficient conditions. Both the bZIP19 and the bZIP23 proteins were found to bind a 10-bp imperfect palindromic sequence, termed the ZDRE. Subsequently, ZDRE motifs were identified in the promoters of *ZIP* transporter genes that are responsive to zinc deficiency. Consistent with the roles bZIP19 and bZIP23 play in zinc deficiency-responsive transcriptional control, zinc deficiency-responsive *ZIP* transporter genes were not found to be induced in the *bzip19 bzip23* double mutant by a zinc deficiency. Orthologues of bZIP19 and bZIP23, their target genes, and the ZDRE motif are conserved in different plant species, indicating that

mechanisms underlying the zinc-deficiency response are conserved in the plant kingdom [147].

26.6 Transcription Factors Involved in Responses to Excess Metals in the Soil

Some metals in the soil are known to induce stress responses. Transcription factors involved in the tolerance to excess aluminum (Al) and cadmium (Cd) in the soil have been identified, suggesting that they could be used in future strategies to develop metal-resistant crops.

26.6.1 Transcription Factors Mediating Al Tolerance

Ionic Al that is produced in acidic soils inhibits root elongation, even at low concentrations. The consequent inhibition of water and nutrient uptake results in a reduction in crop production [148]. One of the best-known mechanisms to tolerate Al is to excrete organic acid anions, which chelate this metal [148]. In the case of *Arabidopsis*, malate is excreted and the expression of a malate transporter gene, *AtALMT1*, is induced in response to Al [149]. *STOP1* (*SENSITIVE TO PROTON RHIZOTOXICITY 1*) encoding a C_2H_2-type zinc finger protein was found to be a regulator of this Al response [150]. An *Arabidopsis* mutant, *stop1*, was originally isolated by its hypersensitivity to proton rhizotoxicity and was found to exhibit shorter roots under Al stress and low pH conditions. On the other hand, another C_2H_2-type zinc finger protein, ART1 (Al resistance transcription factor 1), regulates Al tolerance in rice [151]. Similar to the case of the *stop1* mutant of *Arabidopsis*, the root length of the *art1* mutant was shorter than that of the wild-type rice under Al stress. In response to Al, ART1 induces the expression of Al tolerance genes including *STAR1* and *STAR2*, which encode ATP binding and transmembrane domains of a novel ABC transporter, respectively [151, 152]. The ABC transporter transports UDP glucose, which may be used to modify the cell wall [152]. Neither the *STOP1* transcript levels nor the *ART1* transcript levels were affected by Al treatment, suggesting that the expression of *STOP1* and *ART1* may be posttranscriptionally regulated by the Al conditions. As microarray data have shown that genes downstream of STOP1 are different from those of ART1, except in a few cases, STOP1 and ART1 were suggested to produce Al tolerance via different mechanisms in distinct plant species [151, 153].

26.6.2 The HsfA4a Transcription Factor that Confers Cd Tolerance

Cd is one of the most dangerous heavy metals in the environment. Recently, class A4 heat shock transcription factors (HsfA4a) were found to confer Cd tolerance in rice and wheat (*Triticum aestivum*) [154]. Wheat and rice HsfA4a proteins were identified as factors that conferred Cd tolerance to a Cd-hypersensitive yeast strain. Over-

expression of wheat *HsfA4a* was also found to enhance Cd tolerance in transgenic rice plants, whereas the knockdown of *HsfA4a* in rice increased the sensitivity to Cd, suggesting that *HsfA4a* plays an important role in Cd tolerance in rice. In both rice and wheat, Cd treatment induces the expression of *HsfA4a*, which in turn induces the expression of a gene encoding a metallothionein that is a well-known chelator of Cd. Although further analysis including the identification of additional target genes of HsfA4a might be necessary, phenotypic analysis of transgenic rice plants overexpressing *HsfA4a* has already suggested that the HsfA4a transcription factor will be useful in the future development of Cd-resistant plants to enable the phytoremediation of Cd-contaminated fields.

26.7 Conclusions and Prospects

As described in this chapter, a number of transcription factors involved in abiotic stress responses have been identified over the past decade. Although most were found in *Arabidopsis*, the knowledge obtained using this and other model plants has allowed us to identify functional homologues in commercially important crops and to develop strategies for enhancement of stress tolerance in these plants. Transcription factors associated with stress responses may possess the potential to induce the systematic activation and/or repression of stress-responsive genes in perfect synchrony. Thus, when expression of multiple components in a single cascade or pathway or activation of multiple different cascades or pathways is needed to improve tolerance to a particular stress signal, the utilization of transcription factors could be an ideal strategy. In fact, the expression of Dof1 led to the enhancement of nitrogen assimilation and better growth under nitrogen-deficient conditions, which had not been adequately achieved through the genetic modification of genes for enzymes involved in nitrate reduction and assimilation (Figure 26.2). This is a good example of how the use of transcription factors can improve stress tolerance via the synchronous modification of multiple genes.

The phenotypes of transgenic plants overexpressing some transcription factors have already indicated that it is possible not only to improve tolerance to abiotic stress and increase plant productivity but also to develop metal hyperaccumulator plants for the phytoremediation of contaminated soil or water. Many transcription factors are likely expressed in plant cells. Indeed, more than 1000 genes encoding putative transcription factors have been identified in the *Arabidopsis* genome [155]. However, the functions of most of the plant transcription factors have not yet been determined. Thus, identifying the unknown functions of plant transcription factors may make it possible in the future to generate crops with superior characteristics.

Recent advances in our understanding of the physiological and molecular features of transcription factors associated with abiotic stress suggest that posttranslational control is a key regulatory mechanism in many cases. This agrees with the general hypothesis that the gene expression associated with the primary response is mediated by preexisting transcription factors. Hence, the genetic modification of mechanisms

underlying such posttranscriptional control may be a strategy to improve the stress tolerance of crops. This might be particularly true when the simple overexpression of a transcription factor does not produce the expected phenotype or results in a strong phenotype with negative effects. In fact, when DREB1/CBF was constitutively and strongly expressed under the control of the 35S promoter, growth defects were induced as the stress response in general involves growth arrest. Further analysis of the regulatory mechanisms underlying transcription factor activity control in plants is thus warranted and would expand the opportunities to develop crops with an improved tolerance to environmental stresses in a more sophisticated manner.

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References

1 Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Wakasa, J., Hayashizaki, H., and Shinozaki, K. (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J.*, **31**, 279–292.

2 Vinocur, B. and Altman, A. (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr. Opin. Biotechnol.*, **16**, 123–132.

3 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.*, **57**, 781–803.

4 Zhu, J.-K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.*, **53**, 247–273.

5 Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T., and Shinozaki, K. (1999) A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *Plant Cell*, **11**, 1743–1754.

6 Tran, L.-S.P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA.*, **104**, 20623–20628.

7 Osakabe, Y., Maruyama, K., Seki, M., Satou, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2005) Leucine-rich repeat receptor-like kinase1 is a key membrane-bound regulator of abscisic acid early signaling in *Arabidopsis*. *Plant Cell*, **17**, 1105–1119.

8 Osakabe, Y., Mizuno, S., Tanaka, H., Maruyama, K., Osakabe, K., Todaka, D., Fujita, Y., Kobayashi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2010) Overproduction of the membrane bound receptor-like protein kinase 1, PRK1, enhances abiotic stress tolerance in *Arabidopsis*. *J. Biol. Chem.*, **285**, 9190–9201.

9 Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994) The 5′-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought-, and ABA-regulated gene expression. *Plant Mol. Biol.*, **24**, 701–713.

10 Jiang, C., Iu, B., and Singh, J. (1996) Requirement of a CCGAC *cis*-acting element for cold induction of the *BN115* gene from winter *Brassica napus*. *Plant Mol. Biol.*, **30**, 679–684.

11 Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcription activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA*, **94**, 1035–1040.

12 Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 571–599.

13 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*, **10**, 1391–1406.

14 Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2002) DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem. Biophys. Res. Commun.*, **290**, 998–1009.

15 Nakashima, K., Shinwari, Z.K., Sakuma, Y., Seki, M., Miura, S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000) Organization and expression of two *Arabidopsis DREB2* genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. *Plant Mol. Biol.*, **42**, 657–665.

16 Sakuma, Y., Maruyama, K., Osakabe, Y., Qin, F., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell*, **18**, 1292–1309.

17 Qin, F., Sakuma, Y., Tran, L.-S.P., Maruyama, K., Kidokoro, S., Fujita, Y., Fujita, M., Umezawa, T., Sawano, Y., Miyazono, K., Tanokura, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2008) *Arabidopsis* DREB2A-interacting proteins function as RING E3 ligases and negatively regulate plant drought stress-responsive gene expression. *Plant Cell*, **20**, 1693–1707.

18 Sakuma, Y., Maruyama, K., Qin, F., Osakabe, Y., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) Dual function of an *Arabidopsis* transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. *Proc. Natl. Acad. Sci. USA*, **103**, 18822–18827.

19 Choi, H.-I., Hong, J.-H., Ha, J.-O., Kang, J.-Y., and Kim, S.Y. (2000) ABFs, a family of ABA-responsive element binding factors. *J. Biol. Chem.*, **275**, 1723–1730.

20 Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000) *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc. Natl. Acad. Sci. USA*, **97**, 11632–11637.

21 Kang, J.-y., Choi, H.-i., Im, M.-y., and Kim, S.Y. (2002) *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell*, **14**, 343–357.

22 Kim, S., Kang, J.-y., Cho, D.-I., Park, J.H., and Kim, S.Y. (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *Plant J.*, **40**, 75–87.

23 Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M.M., Seki, M., Hiratsu, K., Ohme-Takagi, M.,

Shinozaki, K., and Yamaguchi-Shinozaki, K. (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell*, **17**, 3470–3488.

24 Furihata, T., Maruyama, K., Fujita, Y., Umezawa, T., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc. Natl. Acad. USA*, **103**, 1988–1993.

25 Koornneef, M., Jorna, M.L., Brinkhorst-van der Swan, D.L.C., and Karssen, C.M. (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. *Theor. Appl. Genet.*, **61**, 385–393.

26 Koornneef, M., Reuling, G., and Karssen, C.M. (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiol. Plant.*, **61**, 377–383.

27 Fujii, H., Verslues, P.E., and Zhu, J.-K. (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. *Plant Cell*, **19**, 485–494.

28 Fujii, H. and Zhu, J.-K. (2009) *Arabidopsis* mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc. Natl. Acad. Sci. USA*, **106**, 8380–8385.

29 Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., Umezawa, T., Fujita, M., Maruyama, K., Ishiyama, K., Kobayashi, M., Nakasone, S., Yamada, K., Ito, T., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2009) Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in *Arabidopsis*. *Plant Cell Physiol.*, **50**, 2123–2132.

30 Nakashima, K., Fujita, Y., Kanamori, N., Katagiri, T., Umezawa, T., Kidokoro, S., Maruyama, K., Yoshida, T., Ishiyama, K., Kobayashi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2009) Three *Arabidopsis* SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant Cell Physiol.*, **50**, 1345–1363.

31 Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., and Shinozaki, K. (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **10**, 17588–17593.

32 Finkelstein, R.R., Gampala, S.S.L., and Rock, C.D. (2002) Abscisic acid signaling in seeds and seedlings. *Plant Cell*, **13**, S15–S25

33 Sheen, J. (1998) Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. *Proc. Natl. Acad. Sci. USA*, **95**, 975–980.

34 Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., and Grill, E. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science*, **324**, 1064–1068.

35 Park, S.-Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T.-F.F., Alfred, S.E., Bonetta, D., Finkelstein, R., Provart, N.J., Desveaux, D., Rodriguez, P.L., McCourt, P., Zhu, J.-K., Schroeder, J.I., Volkman, B.F., and Cutler, S.R. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science*, **324**, 1068–1071.

36 Huang, X.-Y., Chao, D.-Y., Gao, J.-P., Zhu, M.-Z., Shi, M., and Lin, H.-X. (2009) A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. *Genes Dev.*, **23**, 1805–1817.

37 Asano, T., Masuda, D., Yasuda, M., Nakashita, H., Kudo, T., Kimura, M., Yamaguchi, K., and Nishiuchi, T. (2008) *AtNFXL1*, an *Arabidopsis* homologue of the human transcription factor NF-X1, functions as a negative regulator of the

trichothecene phytotoxin-induced defense response. *Plant J.*, **53**, 450–464.
38 Lisso, J., Altmann, T., and Müssig, C. (2006) The *AtNFXL1* gene encodes a NF-X1 type zinc finger protein required for growth under salt stress. *FEBS Lett.*, **580**, 4851–4856.
39 Li, W.-X., Oono, Y., Zhu, J., He, X.-J., Wu, J.-M., Iida, K., Lu, X.-Y., Cui, X., Jin, H., and Zhu, J.-K. (2008) The *Arabidopsis* NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *Plant Cell*, **20**, 2238–2251.
40 Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., and Xiong, L. (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci. USA*, **103**, 12987–12992.
41 Cominelli, E., Galbiati, M., Vavasseur, A., Conti, L., Sala, T., Vuylsteke, M., Leonhardt, N., Dellaporta, S.L., and Tonelli, C. (2005) A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. *Curr. Biol.*, **15**, 1196–1200.
42 Liang, Y.-K., Dubos, C., Dodd, I.C., Holroyd, G.H., Hetherington, A.M., and Campbell, M.M. (2005) *At*MYB61, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. *Curr. Biol.*, **15**, 1201–1206.
43 Ramirez, V., Coego, A., Lopez, A., Agorio, A., Flors, V., and Vera, P. (2009) Drought tolerance in *Arabidopsis* is controlled by the *OCP3* disease resistance regulator. *Plant J.*, **58**, 578–591.
44 Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science*, **280**, 104–106.
45 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.*, **17**, 287–291.
46 Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D., and Thomashow, M.F. (2000) Overexpression of the *Arabidopsis CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.*, **124**, 1854–1865.
47 Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T., and Thomashow, M.F. (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.*, **127**, 910–917.
48 Kasuga, M., Miura, S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004) A combination of the *Arabidopsis* DREB1A gene and stress-inducible *rd29A* promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.*, **45**, 346–350.
49 Hsieh, T.-H., Lee, J.-T., Charng, Y.-Y., and Chan, M.-T. (2002) Tomato plants ectopically expressing *Arabidopsis* CBF1 show enhanced tolerant to water-deficit stress. *Plant Physiol.*, **130**, 618–626.
50 Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol.*, **47**, 141–153.
51 Oh, S.-J., Song, S.I., Kim, Y.S., Jang, H.-J., Kim, S.Y., Kim, M., Kim, Y.-K., Nahm, B.H., and Kim, J.-K. (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiol.*, **138**, 341–351.
52 Pellegrineschi, A., Reynolds, M., Pacheco, M., Brito, R.M., Almeraya, R., Yamaguchi-Shinozaki, K., and Hoisington, D. (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. *Genome*, **47**, 493–500.
53 Qin, F., Sakuma, Y., Li, J., Liu, Q., Li, Y.-Q., Shinozaki, K., and

Yamaguchi-Shinozaki, K. (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. *Plant Cell Physiol.*, **45**, 1042–1052.

54 Zhang, J.Z., Creelman, R.A., and Zhu, J.-K. (2004) From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiol.*, **135**, 615–621.

55 Maruyama, K., Sakuma, Y., Kasuga, M., Ito, Y., Seki, M., Goda, H., Shimada, Y., Yoshida, S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004) Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. *Plant J.*, **38**, 982–993.

56 Sakamoto, H., Maruyama, K., Sakuma, Y., Meshi, T., Iwabuchi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004) *Arabidopsis* Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. *Plant Physiol.*, **136**, 2734–2746.

57 Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.-H., Hong, X., Agarwal, M., and Zhu, J.-K. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.*, **17**, 1043–1054.

58 Lee, B.-H., Henderson, D.A., and Zhu, J.-K. (2005) The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell*, **17**, 3155–3175.

59 Ishitani, M., Xiong, L., Lee, H., Stevenson, B., and Zhu, J.-K. (1998) *HOS1*, a genetic locus involved in cold-responsive gene expression in *Arabidopsis*. *Plant Cell*, **10**, 1151–1162.

60 Miura, K., Jin, J.B., Lee, J., Yoo, C.Y., Stirm, V., Miura, T., Ashworth, E.N., Bressan, R.A., Yun, D.-J., and Hasegawa, P.M. (2007) SIZ1-mediated sumoylation of ICE1 controls *CBF3/DREB1A* expression and freezing tolerance in *Arabidopsis*. *Plant Cell*, **19**, 1403–1414.

61 Dong, C.-H., Agarwal, M., Zhang, Y., Xie, Q., and Zhu, J.-K. (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proc. Natl. Acad. Sci. USA*, **103**, 8281–8286.

62 Agarwal, M., Hao, Y., Kapoor, A., Dong, C.-H., Fujii, H., and Zheng, X. (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J. Biol. Chem.*, **281**, 37636–37645.

63 Thomashow, M.F. (2010) Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiol.*, **154**, 571–577.

64 Doherty, C.J., Van Buskirk, H.A., Myers, S.J., and Thomashow, M.F. (2009) Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *Plant Cell*, **21**, 972–984.

65 Larkindale, J., Hall, J.D., Knight, M.R., and Vierling, E. (2005) Heat stress phenotypes of *Arabidopsis* mutants implicate multiple signaling pathways in the acquisition of thermotolerance. *Plant Physiol.*, **138**, 882–897.

66 Wang, W., Vinocur, B., Shoseyov, O., and Altman, A. (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci.*, **9**, 244–252.

67 Parsell, D.A. and Lindquist, S. (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.*, **27**, 437–496.

68 Schöffl, F., Prändl, R., and Reindl, A. (1998) Regulation of the heat-shock response. *Plant Physiol.*, **117**, 1135–1141.

69 Nover, L., Bharti, K., Döring, P., Mishra, S.K., Ganguli, A., and Scharf, K.-D. (2001) *Arabidopsis* and the heat stress transcription factor world: how many heat stress transcription factors do we need? *Cell Stress Chaperones*, **6**, 177–189.

70 Lohmann, C., Eggers-Schumacher, G., Wunderlich, M., and Schöffl, F. (2004) Two different heat shock transcription factors regulate immediate early expression of stress genes in *Arabidopsis*. *Mol. Genet. Genomics*, **271**, 11–21.

71 Guo, L., Chen, S., Liu, K., Liu, Y., Ni, L., Zhang, K., and Zhang, L. (2008) Isolation of heat shock factor HsfA1a-binding sites *in vivo* revealed variations of heat shock elements in *Arabidopsis thaliana*. *Plant Cell Physiol.*, **49**, 1306–1315.

72 Charng, Y.-Y., Liu, H.-C., Liu, N.-Y., Chi, W.-T., Wang, C.-N., Chang, S.-H., and Wang, T.-T. (2007) A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in *Arabidopsis*. *Plant Physiol.*, **143**, 251–262.

73 Duina, A.A., Kalton, H.M., and Gaber, R.F. (1998) Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of the heat shock response. *J. Biol. Chem.*, **273**, 18974–18978.

74 Morimoto, R.I. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.*, **12**, 3788–3796.

75 Baniwal, S.K., Bharti, K., Chan, K.Y., Fauth, M., Ganguli, A., Kotak, S., Mishra, S.K., Nover, L., Port, M., Scharf, K.-D., Tripp, J., Weber, C., Zielinski, D., and von Koskull-Döring, P. (2004) Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. *J. Biosci.*, **29**, 471–487.

76 Yamada, K., Fukao, Y., Hayashi, M., Fukazawa, M., Suzuki, I., and Nishimura, M. (2007) Cytosolic HSP90 regulates the heat shock response that is responsible for heat acclimation in *Arabidopsis thaliana*. *J. Biol. Chem.*, **282**, 37794–37804.

77 Reindl, A., Schöffl, F., Schell, J., Koncz, C., and Bakó, L. (1997) Phosphorylation by a cyclin-dependent kinase modulates DNA binding of the *Arabidopsis* heat-shock transcription factor HSF1 *in vitro*. *Plant Physiol.*, **115**, 93–100.

78 Schramm, F., Larkindale, J., Kiehlmann, E., Ganguli, A., Englich, G., Vierling, E., and Von Koskull-Döring, P. (2008) A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of *Arabidopsis*. *Plant J.*, **53**, 264–274.

79 Yoshida, T., Sakuma, Y., Todaka, D., Maruyama, K., Qin, F., Mizoi, J., Kidokoro, S., Fujita, Y., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2008) Functional analysis of an *Arabidopsis* heat-shock transcription factor *HsfA3* in the transcriptional cascade downstream of the DREB2A stress-regulatory system. *Biochem. Biophys. Res. Commun.*, **368**, 515–521.

80 Larkindale, J. and Vierling, E. (2008) Core genome responses involved in acclimation to high temperature. *Plant Physiol.*, **146**, 748–761.

81 Novillo, F., Alonso, J.M., Ecker, J.R., and Salinas, J. (2004) CBF2/DREB1C is a negative regulator of *CBF1/CREB1B* and *CBF3/DREB1A* expression and plays a central role in stress tolerance in *Arabidopsis*. *Proc. Natl. Acad. USA*, **101**, 3985–3990.

82 Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F., and Zhang, J.Z. (2002) Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. *Plant Physiol.*, **130**, 639–648.

83 Scheible, W.R., González-Fontes, A., Lauerer, M., Müller-Röber, B., Caboche, M., and Stitt, M. (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell*, **9**, 783–798.

84 Wang, R., Tischner, R., Gutiérrez, R.A., Hoffman, M., Xing, X., Chen, M., Coruzzi, G., and Crawford, N.M. (2004) Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. *Plant Physiol.*, **136**, 2512–2522.

85 Wang, R., Xing, X., and Crawford, N. (2007) Nitrite act as a transcriptome signal as micromolar concentrations in *Arabidopsis* roots. *Plant Physiol.*, **145**, 1735–1745.

86 Sugiharto, B., Suzuki, I., Burnell, J.N., and Sugiyama, T. (1992) Glutamine induces the N-dependent accumulation of mRNAs encoding

phospho*enol*pyruvate carboxylase and carbonic anhydrase in detached maize leaf tissue. *Plant Physiol.*, **100**, 2066–2070.

87 Vincentz, M., Moureaux, T., Leydecker, M.T., Vaucheret, H., and Caboche, M. (1993) Regulation of nitrate and nitrite reductase expression in *Nicotiana plumbaginifolia* leaves by nitrogen and carbon metabolites. *Plant J.*, **3**, 315–324.

88 Lin, X., Wang, S., Zhang, J., Feng, Q., Zhang, L., Fan, D., Li, X., Yuan, D., Han, B., and Zhang, Q. (2006) Expression profiles of 10, 422 genes at early stage of low nitrogen stress in assayed using a cDNA microarray. *Plant Mol. Biol.*, **60**, 617–631.

89 Wang, R., Okamoto, M., Xing, X., and Crawford, N.M. (2003) Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. *Plant Physiol.*, **132**, 556–567.

90 Scheible, W.-R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., Schindelasch, D., Thimm, O., Udvardi, M.K., and Stitt, M. (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. *Plant Physiol.*, **136**, 2483–2499.

91 Konishi, M. and Yanagisawa, S. (2010) Identification of a nitrate-responsive *cis*-element in the *Arabidopsis NIR1* promoter defines the presence of multiple *cis*-regulatory elements for nitrogen response. *Plant J.*, **63**, 269–282.

92 Zhang, H. and Forde, B.G. (1998) An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. *Science*, **279**, 407–409.

93 Gan, Y., Filleur, S., Rahman, A., Gotensparre, S., and Forde, B.G. (2005) Nutritional regulation of *ANR1* and other root-expressed MADS-box genes in *Arabidopsis thaliana*. *Planta*, **222**, 730–742.

94 Rubio, V., Linhares, F., Solano, R., Martin, A.C., Iglesias, J., Leyva, A., and Paz-Ares, J. (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Gene Dev.*, **15**, 2122–2133.

95 Todd, C.D., Zeng, P., Huete, A.M.R., Hoyos, M.E., and Polacco, J.C. (2004) Transcripts of MYB-like genes respond to phosphorous and nitrogen deprivation in *Arabidopsis*. *Planta*, **219**, 1003–1009.

96 Castaings, L., Camargo, A., Pocholle, D., Gaudon, V., Texier, Y., Boutet-Mercey, S., Taconnat, L., Renou, J.-P., Daniel-Vedele, F., Fernandez, E., Meyer, C., and Krapp, A. (2009) The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. *Plant J.*, **57**, 426–435.

97 Rubin, G., Tohge, T., Matsuda, F., Saito, K., and Scheible, W.-R. (2009) Members of the *LBD* family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in *Arabidopsis*. *Plant Cell*, **21**, 3567–3584.

98 Tohge, T., Nishiyama, Y., Hirai, M.Y., Yano, M., Nakajima, J., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D.B., Kitayama, M., Noji, M., Yamazaki, M., and Saito, K. (2005) Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. *Plant J.*, **42**, 218–235.

99 Bi, Y.-M., Zhang, Y., Signorelli, T., Zhao, R., Zhu, T., and Rothstein, S. (2005) Genetic analysis of *Arabidopsis* GATA transcription factor gene family reveals a nitrae-inducible member important for chlorophyll synthesis and glucose sensitivity. *Plant J.*, **44**, 680–692.

100 Coruzzi, G.M. and Zhou, L. (2001) Carbon and N sensing and signaling in plants: emerging "matrix effects." *Curr. Opin. Plant Biol.*, **4**, 247–253.

101 Yanagisawa, S. (2002) The Dof family of plant transcription factors. *Trends Plant Sci.*, **7**, 555–560.

102 Yanagisawa, S., Akiyama, A., Kisaka, H., Uchimiya, H., and Miwa, T. (2004) Metabolic engineering with Dof1 transcription factor in plants: improved nitrogen assimilation and growth under low-nitrogen conditions. *Proc. Natl. Acad. Sci. USA*, **101**, 7833–7838.

103 Bari, R., Pant, B.D., Stitt, M., and Scheible, W.-R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol.*, **141**, 988–999.

104 Nilsson, L., Müller, R., and Nielsen, T.H. (2007) Increased expression of the MYB-related transcription factor, *PHR1*, leads to enhanced phosphate uptake in *Arabidopsis thaliana*. *Plant Cell Environ.*, **30**, 1499–1512.

105 Bustos, R., Castrillo, G., Linhares, F., Puga, M.I., Rubio, V., Perez-Perez, J., Solano, R., Leyva, A., and Paz-Ares, J. (2010) A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in *Arabidopsis*. *PLoS Genet.*, **6** (9), e1001102. doi: 10.1371/journal.pgen.1001102

106 Misson, J., Raghothama, K.G., jain, A., Jouhet, J., Block, M.A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N., Doumas, P., Nacry, P., Herrerra-Estrella, L., Nussaume, L., and Thiboud, M.-C. (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. *Proc. Natl. Acad. Sci. USA*, **102**, 11934–11939.

107 Müller, R., Morant, M., jarmer, H., Nilsson, L., and Nielsen, T.H. (2007) Genome-wide analysis of the *Arabidopsis* leaf transcriptome reveals interaction of phosphate and sugar metabolism. *Plant Physiol.*, **143**, 156–171.

108 Zhou, J., Jiao, F., Wu, Z., Li, Y., Wang, X., He, X., Zhong, W., and Wu, P. (2008) *OsPHR2* is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. *Plant Physiol.*, **146**, 1673–1686.

109 Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A.S., Raghothama, K.G., Beak, D., Koo, Y.D., Jin, J.B., Bressan, R.A., Yun, D.-J., and Hasegawa, P.M. (2005) The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proc. Natl. Acad. Sci. USA*, **102**, 7760–7765.

110 Aung, K., Lin, S.-I., Wu, C.-C., Huang, Y.-T., Su, C.-I., and Chiou, T.-J. (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsensemutation in a microRNA399 target gene. *Plant Physiol.*, **141**, 1000–1011.

111 Shin, H., Shin, H.-S., Chen, R., and Harrison, M.J. (2006) Loss of *At4* function impacts phosphate distribution between the roots and the shoots during phosphate starvation. *Plant J.*, **45**, 712–726.

112 Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A., and Paz-Ares, J. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat. Genet.*, **39**, 1033–1037.

113 Doerner, P. (2008) Phosphate starvation signaling: a threesome controls systemic Pi homeostasis. *Curr. Opin. Plant Biol.*, **11**, 536–540.

114 Wu, P., Ma, L., Hu, X., Wang, M., Wu, Y., Liu, F., and Deng, X.W. (2003) Phosphate starvation triggers distinct alterations of genome expression in *Arabidopsis* roots and leaves. *Plant Physiol.*, **132**, 1260–1271.

115 Devaiah, B.N., Madhuvanthi, R., Karthikeyan, A.S., and Raghothama, K.G. (2009) Phosphate starvation responses and gibberellic acid biosynthesis are regulated by the *MYB62* transcription factor in *Arabidopsis*. *Mol. Plant*, **2**, 43–58.

116 Devaiah, B.N., Nagarajan, V.K., and Raghothama, K.G. (2007) Phosphate homeostasis and root development in *Arabidopsis* are synchronized by the zinc finger transcription factor ZAT6. *Plant Physiol.*, **145**, 147–159.

117 Devaiah, B.N., Karthikeyan, A.S., and Raghothama, K.G. (2007) WRKY75 transcription factor is a modulator of

phosphate acquisition and root development in *Arabidopsis*. *Plant Physiol.*, **143**, 1789–1801.

118 Chen, Y.-F., Li, L.-Q., Xu, Q., Kong, Y.-H., Wang, H., and Wu, W.-H. (2009) The WRKY6 transcription factor modulates *PHOSPHATE1* expression in response to low Pi stress in *Arabidopsis*. *Plant Cell*, **21**, 3554–3566.

119 Humburger, D., Rezzonico, E., Petétot, J.M.-C., Somerville, C., and Poirier, Y. (2002) Identification and characterization of the *Arabidopsis PHO1* gene involved in phosphate loading to the xylem. *Plant Cell*, **14**, 889–902.

120 Chen, Z.-H., Nimmo, G.A., Jenkins, G.I., and Nimmo, H.G. (2007) BHLH32 modulates several biochemical and morphological processes that respond to Pi starvation in *Arabidopsis*. *Biochem. J.*, **405**, 191–198.

121 Yi, K., Wu, Z., Zhou, J., Du, L., Guo, L., Wu, Y., and Wu, P. (2005) *OsPTF1*, a novel transcription factor involved in tolerance to phosphate starvation in rice. *Plant Physiol.*, **138**, 2087–2096.

122 Maruyama-Nakashita, A., Nakamura, Y., Yamaya, T., and Takahashi, H. (2004) Regulation of high-affinity sulfate transporters in plants: towards systematic analysis of sulfur signaling and regulation. *J. Exp. Bot.*, **55**, 1843–1849.

123 Maruyama-Nakashita, A., Nakamura, Y., Watanabe-Takahashi, A., Inoue, E., Yamaya, T., and Takahashi, H. (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in *Arabidopsis* roots. *Plant J.*, **42**, 305–314.

124 Maruyama-Nakashita, A., Nakamura, Y., Tohge, T., Saito, K., and Takahashi, H. (2006) *Arabidopsis* SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. *Plant Cell*, **18**, 3235–3251.

125 Guo, H. and Ecker, J. (2004) The ethylene signaling pathway: new insights. *Curr. Opin. Plant Biol.*, **7**, 40–49.

126 Kataoka, T., Watanabe-Takahashi, A., Hayashi, N., Ohnishi, M., Mimura, T., Buchner, P., Hawkesford, M.J., Yamaya, T., and Takahashi, H. (2004) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in *Arabidopsis*. *Plant Cell*, **16**, 2693–2704.

127 Kawashima, C.G., Berkowitz, O., Hell, R., Noji, M., and Saito, K. (2005) Characterization and expression analysis of a serine acetyltransferase gene family involved in a key step of the sulfur assimilation pathway in *Arabidopsis*. *Plant Physiol.*, **137**, 220–230.

128 Halkier, B.A. and Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol.*, **57**, 303–333.

129 Römheld, V. (1987) Different strategies for iron acquisition in higher plants. *Physiol. Plant.*, **70**, 231–234.

130 Ling, H.-Q., Baur, P., Bereczky, Z., Keller, B., and Ganal, M. (2002) The tomato *fer* gene encoding a HLH protein controls iron-uptake responses in roots. *Proc. Natl. Acad. Sci. USA*, **99**, 13938–13943.

131 Brumbarova, T. and Bauer, P. (2005) Iron-mediated control of the basic helix–loop–helix protein FER, a regulator of iron uptake in tomato. *Plant Physiol.*, **137**, 1018–1026.

132 Colangelo, E.P. and Guerinot, M.L. (2004) The essential basic helix–loop–helix protein FIT1 is required for the iron deficiency response. *Plant Cell*, **16**, 3400–3412.

133 Jakoby, M., Wang, H.-Y., Reidt, W., Weisshaar, B., and Bauer, P. (2004) *FRU* (*BHLH029*) is required for induction of iron mobilization genes in *Arabidopsis thaliana*. *FEBS Lett.*, **577**, 528–534.

134 Yuan, Y., Wu, H., Wang, N., Li, J., Zhao, W., Du, J., Wang, D., and Ling, H.-Q. (2008) FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in *Arabidopsis*. *Cell Res.*, **18**, 385–397.

135 Wang, H.-Y., Klatte, M., Jakoby, M., Baumlein, H., Weisshaar, B., and Baur, P. (2007) Iron deficiency-mediated stress regulation of four subgroup Ib *BHLH* genes in *Arabidopsis thaliana*. *Planta*, **226**, 897–908.

136 Ogo, Y., Itai, R.N., Nakanishi, H., Inoue, H., Kobayashi, T., Suzuki, M., Takahashi, M., Mori, S., and Nishizawa, N.K. (2006)

Isolation and characterization of IRO2, a novel iron-regulated bHLH transcription factor in graminaceous plants. *J. Exp. Bot.*, **57**, 2867–2878.

137 Ogo, Y., Itai, R.N., Nakanishi, H., Kobayashi, T., Takahashi, M., Mori, S., and Nishizawa, N.K. (2007) The rice bHLH protein OsIRO2 is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions. *Plant J.*, **51**, 366–377.

138 Inoue, H., Kobayashi, T., Nozoye, T., Takahashi, M., Kakei, Y., Suzuki, K., Nakazono, M., Nakanishi, H., Mori, S., and Nishizawa, N.K. (2009) Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. *J. Biol. Chem.*, **284**, 3470–3479.

139 Kobayashi, T., Ogo, Y., Itai, R.N., Nakanishi, H., Takahashi, M., Mori, S., and Nishizawa, N.K. (2007) The transcription factor IDEF1 regulates the response to and tolerance of iron deficiency in plants. *Proc. Natl. Acad. Sci. USA*, **104**, 19150–19155.

140 Kobayashi, T., Nakayama, Y., Itai, R.N., Nakanishi, H., Yoshihara, T., Mori, S., and Nishizawa, N.K. (2003) Identification of novel *cis*-acting elements, IDE1 and IDE2, of the barley *IDS2* gene promoter conferring iron-deficiency-inducible, root-specific expression in heterogeneous tobacco plants. *Plant J.*, **36**, 780–793.

141 Kobayashi, T., Itai, R.N., Ogo, Y., Kakei, Y., Nakanishi, H., Takahashi, M., and Nishizawa, N.K. (2009) The rice transcription factor IDEF1 is essential for the early response to iron deficiency, and induces vegetative expression of late embryogenesis abundant genes. *Plant J.*, **60**, 948–961.

142 Koike, S., Inoue, H., Mizuno, D., Takahashi, M., Nakanishi, H., Mori, S., and Nishizawa, N.K. (2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant J.*, **39**, 415–424.

143 Ogo, Y., Kobayashi, T., Itai, R.N., Nakanishi, H., Kakei, Y., Takahashi, M., Toki, S., Mori, S., and Nishizawa, N.K. (2008) A novel NAC transcription factor, IDEF2, that recognizes the iron deficiency-responsive element 2 regulates the genes involved in iron homeostasis in plants. *J. Biol. Chem.*, **283**, 13407–13417.

144 Vallee, B.L. and Auld, D.S. (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry*, **29**, 5647–5659.

145 Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M.L., and Eide, D. (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proc. Natl. Acad. Sci. USA*, **95**, 7220–7224.

146 Wintz, H., Fox, T., Wu, Y.-Y., Feng, V., Chen, W., Chang, H.-S., Zhu, T., and Vulpe, C. (2003) Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. *J. Biol. Chem.*, **278**, 47644–47653.

147 Assunção, A.G.L., Herrero, E., Lin, Y.-F., Huettel, B., Talukdar, S., Smaczniak, C., Immink, R.G.H., van Eldik, M., Fiers, M., Schat, H., and Aarts, M.G.M. (2010) *Arabidopsis thaliana* transcription factors bZIP19 and bZIP23 regulate the adaptation to zinc deficiency. *Proc. Natl. Acad. Sci. USA*, **107**, 10296–10301.

148 Ma, J.F. (2007) Syndrome of aluminum toxicity and diversity of aluminum resistance in higher plants. *Int. Rev. Cytol.*, **264**, 225–252.

149 Hoekenga, O.A., Maron, L.G., Piñeros, M.A., Cancada, G.M.A., Shaff, J., Kobayashi, Y., Ryan, P.R., Dong, B., Delhaize, E., Sasaki, T., Matsumoto, H., Yamamoto, Y., Koyama, H., and Kochian, L.V. (2006) *AtALMT1*, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in *Arabidopsis*. *Proc. Natl. Acad. USA*, **103**, 9738–9743.

150 Iuchi, S., Koyama, H., Iuchi, A., Kobayashi, Y., Kitabayashi, S., Kobayashi, Y., Ikka, T., Hirayama, T., Shinozaki, K., and Kobayashi, M. (2007) Zinc finger protein STOP1 is critical for proton tolerance in *Arabidopsis* and coregulates a key gene in aluminum tolerance. *Proc. Natl. Acad. USA*, **104**, 9900–9905.

151 Yamaji, N., Huang, C.F., Nagao, S., Yano, M., Sato, Y., Nagamura, Y., and Ma, J.F. (2009) A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. *Plant Cell*, **21**, 3339–3349.

152 Huang, C.F., Yamaji, N., Mitani, N., Yano, M., Nagamura, Y., and Ma, J.F. (2009) A bacterial-type ABC transporter is involved in aluminum tolerance in rice. *Plant Cell*, **21**, 655–667.

153 Sawaki, Y., Iuchi, S., Kobayashi, Y., Kobayashi, Y., Ikka, T., Sakurai, N., Fujita, M., Shinozaki, K., Shibata, D., Kobayashi, M., and Koyama, H. (2009) STOP1 regulates multiple genes that protect *Arabidopsis* from proton and aluminum toxicities. *Plant Physiol.*, **150**, 281–294.

154 Shin, D., Hwang, J.-U., Lee, J., Lee, S., Choi, Y., An, G., Matrinoia, E., and Lee, Y. (2009) Orthologs of the class A4 heat shock transcription factor HsfA4a confer cadmium tolerance in wheat and rice. *Plant Cell*, **21**, 4031–4043.

155 Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C.-Z., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., and Yu, G.-L. (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science*, **290**, 2105–2110.

27
Polyamines in Developing Stress-Resistant Crops

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Polyamines (PAs) are small protonated compounds with key roles in plant development and physiological processes. PAs may also function as stress messengers in plant responses to different stress signals. Recent studies using exogenous application of polyamines and more contemporary genetic manipulation of polyamine levels in crops and model species point to their involvement in stress protection. The different mechanisms by which polyamines exert their functions are presently being unraveled and involve different modes of action that are summarized in this chapter. Polyamines are integrated with other stress-related hormone pathways, such as abscisic acid (ABA), reactive oxygen species (ROS) signaling, nitric oxide, and regulation of ion channels that are now being elucidated. Also, polyamines are implicated in the transcriptional regulation to abiotic and biotic stresses as revealed in recent global transcriptome analyses. The genetic manipulation of polyamine levels has been proven to be an efficient tool for enhancing stress tolerance in many plant species. A number of examples and their potential application to crops for a sustainable agriculture are discussed in this chapter, along with the most recent advances in our understanding of the regulation and mode of action of polyamines.

27.1
Introduction

27.1.1
PA Biosynthesis and Catabolism in Plants

Plants live in an ever-changing and often unpredictable environment that represents the major limiting factors for agricultural crop productivity. Plants, unlike animals, cannot move and therefore encounter a variety of environmental stresses throughout their life cycle. It is predicted that the environmental stresses will become more intense and frequent with climate change, especially global warming. Among abiotic stresses, cold, heat, salinity, and drought adversely affect plant growth and productivity and restrict the crops to reach their full genetic potential [1].

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World population is also increasing at an alarming rate and expected to reach around 10 billion by 2050, which will witness serious food shortages. Therefore, reducing crop loss is a major challenge to meet the increasing food demand [2]. Plants employ various strategies to cope with the ever-changing environmental fluctuations [3].

Polyamines (PAs) are a group of polycationic amine-containing compounds whose most predominant forms are the diamine putrescine (Put), triamine spermidine (Spd), and tetramine spermine (Spm) that play a pivotal role in the regulation of developmental and physiological processes in plants [4]. Put, Spd, and Spm molecules differ in the number of aminopropyl moieties added to the carbon skeleton of Put, and thus they differ in their number of positively charged amine groups at the physiological pH of the cell. Metabolic studies indicate that the intracellular levels of PAs in plants are mostly regulated by anabolic and catabolic processes (Figure 27.1), as well as by their conjugation to hydroxycinnamic acids and other macromolecules such as proteins and DNA.

The PA biosynthetic pathway starts with the synthesis of the diamine Put. In mammals and fungi, Put biosynthesis is exclusively derived from ornitine (Orn) decarboxylation in a reaction catalyzed by ornithine decarboxylase (ODC). Plants and bacteria can also synthesize Put via an alternative pathway involving arginine (Arg) decarboxylation by arginine decarboxylase (ADC), as well as two additional successive

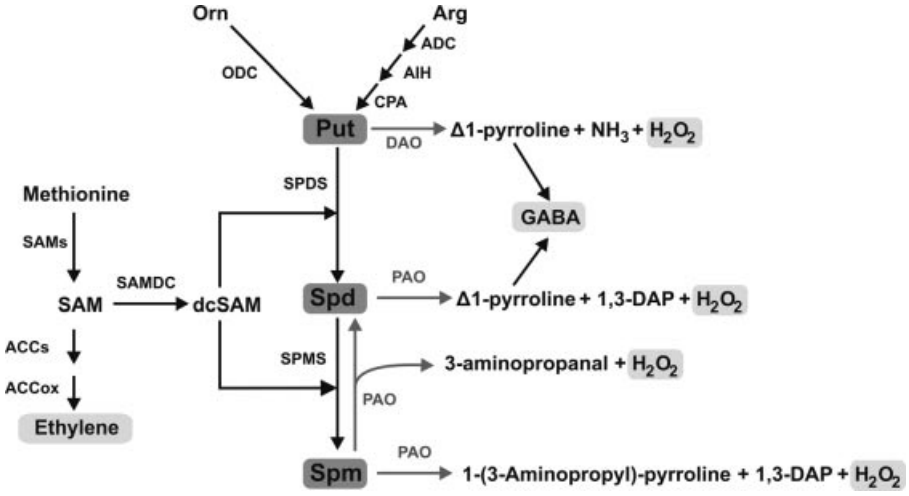

Figure 27.1 Polyamine biosynthesis and catabolism in plants. Biosynthetic pathways are indicated in black and degradation routes in grey. ACCs, ACC synthase; ACCox, ACC oxidase; ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; DAO, diamine oxidase; 1,3-DAP, 1,3-diaminopropane; dcSAM, decarboxylated *S*-adenosylmethionine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SAM, *S*-adenosylmethionine; SAMs, *S*-adenosylmethionine synthase; SAMDC, *S*-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase.

steps involving agmatine iminohydrolase (AIH) and *N*-carbamoylputrescine amidohydrolase (CPA) activities. Put serves as a precursor of Spd and Spm by consecutive additions of aminopropyl groups catalyzed, respectively, by Spd synthase (SPDS) and Spm synthase (SPMS). Both enzymes use decarboxylated *S*-adenosylmethionine (dcSAM) as donor of aminopropyl moieties, which is formed by decarboxylation of SAM in a reaction catalyzed by SAM decarboxylase (SAMDC) (Figure 27.1).

PAs are degraded by oxidative deamination in reactions catalyzed by amine oxidases, in particular diamine oxidases (DAOs) and PA oxidases (PAOs). DAOs display high affinity for diamines, similar to Put, producing Δ^1-pyrroline, H_2O_2, and ammonia (Figure 27.1). Δ^1-pyrroline is catabolized into γ-aminobutyric acid (GABA) (Figure 27.1), which is ultimately converted into succinic acid, a component of the Krebs cycle. PAOs oxidize secondary amine groups from Spd and Spm, leading to the formation of 4-aminobutanal or (3-aminopropyl)-4-aminobutanal, along with 1,3-diaminopropane (DAP) and H_2O_2 (Figure 27.1). Spm could also be backconverted to Spd by PAOs with concomitant production of 3-aminopropanal and H_2O_2. Therefore, the PA metabolic pathway is also interconnected with other metabolic routes involved in the formation of various signaling molecules and metabolites that are relevant to plant stress responses such as ethylene, GABA, or H_2O_2 (Figure 27.1) [5].

Many evidences point to the requirement of Put and Spd for plant life and development. Depletion of Put and Spd levels by genetic or chemical means is lethal in yeast, protists, and plants [6–9]. Indeed, all living organisms analyzed so far contain endogenous pools of Put and Spd. Conversely, Spm-deficient organisms seem viable but show different degrees of dysfunction, thus suggesting an important involvement of Spm in growth and developmental processes [10–13].

In plants, PAs have been implicated in a wide array of fundamental processes such as cell cycle, transcriptome regulation, hormone signaling, plant growth and development, and response to biotic and abiotic stresses [14–21].

27.2 PAs and Stress

The first observation on the effects of stress in PA levels in plants was reported by Richards and Coleman [22], who showed an increase in the endogenous levels of Put in oat plants grown under potassium starvation. Since then, a large number of studies have shown an increase in PA levels in response to different biotic and abiotic stresses [14, 16, 23]. Stress-triggered PA accumulation correlates with enhanced tolerance to different stresses, such as salinity [24–27], chilling [27, 28], osmotic and acidic stresses [29], radiation-induced oxidative stress [30], and so on [23].

Also, early studies based on the application of exogenous PAs or PA biosynthesis inhibitors have also been useful to identify correlation between PA stress accumulation and plant tolerance [16]. Nevertheless, it has to be noted that exogenous application of PAs may have certain limitations, such as differences in the uptake rates between replicates, the possible deleterious effects of PAs when applied to

membranes at high doses, and an insufficient specificity of the inhibitor applied in some instances, determined frequently by differences in the localization of the inhibitor and the target enzyme [31].

Since the identification of the oat *ADC* by Bell and Malmberg [32], many genes coding for enzymes involved in PA metabolism have been cloned from several plant species and their expression under stress conditions analyzed [14, 33]. Reports from those experiments show that some of the PA biosynthetic genes raise their expression levels in response to stress, although with different kinetics. Some PA biosynthetic genes are rapidly induced shortly after stress treatment and undergo a continuous rise or a minor change with a prolonged period of stress. Conversely, others are induced only when the stress is exerted for a certain period. These observations indicate a differential regulation of PA biosynthetic genes during stress, consistent with different pathways involved in the regulation of PA biosynthesis under stress [34]. When trying to combine transcriptional profiles from independent experimental designs, the different kinetics may depend on several factors, such as plant species, duration, and intensity of stress or stress sensitivity of the experimental materials [24, 25, 35, 36]. Hence, rather than analyzing single genes in the PA pathway, a broader approach that aims to analyze the whole PA biosynthetic transcriptome is more informative for the study of gene kinetics. Unfortunately, there are very few examples where these approaches have been undertaken [34, 37–40].

27.3 Transgenic Modifications of PA Biosynthetic Route and Improvement of Stress Tolerance

The identification and cloning of the genes coding for PA biosynthetic enzymes has also allowed the generation of transgenic plants with altered endogenous PA levels, in order to overcome the problems arising from the use of exogenous PAs or the lack of specificity of certain PA biosynthetic inhibitors. Table 27.1 summarizes a number of examples of overexpression of *ODC*, *ADC*, *SAMDC*, and *SPDS* in rice, tobacco, pear, sweet potato, and *Arabidopsis* during over the years, with different results concerning the modification of one or more specific PAs, but all showing in common an enhanced tolerance against a broad spectrum of stress conditions (Table 27.1). Enhanced tolerance always correlated with elevated levels of Put and/or Spd and Spm. Transgenic rice plants carrying oat *ADC* under control of an abscisic acid (ABA)-inducible promoter showed higher ADC activity, higher Put level, and increased biomass under salt stress than wild-type plants [41]. Similarly, constitutive overexpresssion of *Datura stramonium ADC* gene in rice produced transgenic plants that accumulated higher levels of Spd and Spm than the wild type when exposed to drought stress. These lines also showed an improvement in drought tolerance, with a lower degree of chlorophyll loss and leaf curling than the wild type [29]. More recently, constitutive homologous overexpression of *ADC* genes in *Arabidopsis* also leads to transgenic plants with elevated Put levels and resistant to freezing conditions [42] and

Table 27.1 Abiotic stress tolerance in plants with overproduction of PAs obtained by transgenic modification.

Gene	Source	Transgenic plant	Overexpression	Overproduction	Tolerance	Reference
ADC	Oat	Rice	Inducible	Put	Salt	[41]
	D. stramonium	Rice	Constitutive	Spd and Spm	Drought	[29]
	Arabidopsis (*ADC1*)	*Arabidopsis*	Constitutive	Put	Freezing	[42]
	Arabidopsis (*ADC2*)	*Arabidopsis*	Constitutive	Put	Drought	[43]
ODC	Mouse	Tobacco	Constitutive	Put	Salt	[44]
SAMDC	*Tritordeum*	Rice	Inducible	Spd and Spm	Salt	[26]
	Human	Tobacco	Constitutive	Put and Spd	Salt, osmotic	[45]
	Carnation	Tobacco	Constitutive	Put, Spd, and Spm	Broad spectrum	[46]
	Yeast	Tomato	Constitutive	Spd and Spm	Heat	[47]
	Arabidopsis (*SAMDC1*)	*Arabidopsis*	Constitutive	Spm	Salt	[67]
SPDS	*C. ficifolia*	*Arabidopsis*	Constitutive	Spd	Broad spectrum	[48]
	C. ficifolia	Sweet potato	Constitutive	Spd	Broad spectrum	[49]
	Apple	Pear	Constitutive	Spd	Heavy metal (Al)	[50]
ACCs	Carnation	Tobacco	Antisense	Put and Spd	Broad spectrum	[51]
ACCox	Carnation	Tobacco	Antisense	Put and Spd	Broad spectrum	[51]

Source: Adapted from Refs [4, 5].

drought stress [43]. Salt tolerance has also been obtained in tobacco plants overexpressing mouse *ODC*, which showed higher Put levels, a better germination frequencies, and a less degree of wilting in salt than wild-type plants [44].

SAMDC cDNAs have been also used to generate transgenic plants with elevated PA levels (Table 27.1). Constitutive overexpression of *Tritordeum SAMDC* gene in rice resulted in a three- to fourfold increase in Spd and Spm levels in the transformed plants [26]. Stress tolerance of those plants was demonstrated by their normal growth and development under NaCl stress. The tobacco plants obtained by Waie and Rajam [45] are another example of heterologous constitutive *SAMDC* overexpression where a human *SAMDC* gene was driven by a constitutive *CaMV35S* promoter. The transgenic tobacco lines obtained showed higher Spd and Put levels, as well as tolerance to salt and drought stresses [45]. More recently, transgenic tobacco plants overexpressing carnation *SAMDC* also showed a broad-spectrum tolerance to abiotic stresses [46]. Transgenic tomato plants with high levels of Spm and Spd and tolerant to heat stress have been obtained by constitutive overexpression of yeast *SAMDC* gene [47]. Also, homologous overexpression of *SAMDC1* gene in *Arabidopsis* leads to elevated Spm levels and enhanced tolerance to salt stress [67].

Transgenic plants overexpressing *SPDS* share common features of elevated Spd levels as well as broad-spectrum stress tolerance (Table 27.1). Thus, overexpression of *SPDS* from *Cucurbita ficifolia* in *Arabidopsis* enhanced tolerance to chilling, freezing, drought, salinity, osmosis, and paraquat [48]. Transformation of the same gene into sweet potato (*Ipomoea batatas*) produced transgenic plants with more tolerance to salt and drought than the wild type [49]. Higher Spd titers are also found in transgenic pear plants overexpressing apple *MDSPDS1* gene, being also more tolerant than wild-type plants when exposed to $AlCl_3$ long-term stress [50].

On the other hand, Wi and Park [51] employed an alternative way to raise PA levels by favoring the flux of SAM to PAs using antisense silencing of ethylene biosynthesis genes *ACC synthase* and *ACC oxidase* in tobacco. Transgenic plants obtained raised Put and Spd levels and increased their tolerance to oxidative, high salinity, and acid stresses [51]. Previously, high PA levels were also found in a tobacco DFMO-resistant line, which was also resistant to acidic stress conditions [52].

In summary, all these examples show the correlation between accumulation of Put and Spd and Spm with stress tolerance, often with a broad spectrum of stresses. This correlation is also reinforced by the results obtained from loss-of-function mutations in PA biosynthetic genes. For example, EMS mutants of *Arabidopsis thaliana spe1-1* and *spe2-1* (which map to *ADC2*) showing reduced ADC activity are deficient in PA accumulation after acclimation to high NaCl concentrations and exhibit more sensitivity to salt stress [53]. Another study shows that a Ds insertion mutant (*adc2-1*), with Put levels diminished up to 75% of wild-type plants, is more sensitive to salt stress, whereas its salt-induced injury is partly reverted by the addition of exogenous Put [54]. Other *ADC1* (*adc1-2*, *adc1-3*) and *ADC2* (*adc2-3*, *adc2-4*) mutant alleles are more sensitive to freezing, and this phenotype is partially rescued by adding exogenous Put [37]. On the other hand, *acl5/spms Arabidopsis* double mutants that do not produce Spm and thermospermine are hypersensitive to salt and drought stresses, and the phenotype is mitigated by application of exogenous Spm [55].

27.4
Possible Mechanisms of PA Action in Stress Responses

Taken together, results presented in the above sections indicate that elevated PA levels represent a stress-induced protective response with a protective role. However, the precise mechanism of action by which PAs could protect plants from challenging environmental conditions remains unclear, although some progress has been made [4, 56].

Classically, the most common explanation for protective roles of PAs have been related to their chemical structure: the polycationic nature of PAs at physiological pH enables them to modulate ion balance of the cell and interact with anionic molecules, such as DNA, RNA, proteins, and membrane lipids [57, 58]. PA binding to proteins or nucleic acids could not only protect them from degradation but also provide a molecule with the most stable conformation under stress conditions. A large number of evidences suggested that exogenous application of PAs (di-, tri-, and tetraamines) were shown to stabilize plant cell membranes, protecting them from damage under stress conditions [33, 59], and endogenous PAs are also suggested to participate in sustaining membrane integrity [60].

Also, an antioxidative role has been proposed for PAs due to a combination of their possible role as radical-scavenging molecules by means of their dual anion and cation binding properties [61] and their capability to inhibit both lipid peroxidation [62] and metal-catalyzed oxidative reactions [63]. Spm, Spd, and Put all reduce level of superoxide radicals generated by senescing plant cells [64]. Alternatively, PA catabolism produces H_2O_2, a signaling molecule that can enter the stress signal transduction chain promoting an activation of an antioxidative defense response. Owing to the fact that this peroxide production could also be a source of oxidant species, the role of PAs acting as antioxidants is still a matter of debate [23].

On the other hand, more recent data show that modification of endogenous PA levels alters the expression of an important number of genes, most of them stress related. Some of these stress-related genes could promote the synthesis of more protective compounds and render stress tolerance. This is seen, for example, in tobacco *SAMDC* overexpressing plants that have higher levels of expression of several antioxidant enzymes, such as ascorbate peroxidase, superoxide dismutase, and glutathione *S*-transferase [46]. Microarray analysis of *Arabidopsis* plants overexpressing *SPDS* shows that those transgenic plants have higher expression levels of some stress-related transcription factors, such as DREB, WRKY, B-box zinc finger proteins, NAM proteins, and MYB, along with stress-regulated genes, such as low-temperature-induced protein 78 (LTI78 or rd29A) [48]. More recently, analysis of transcriptome profiles of *Arabidopsis* plants overexpressing homologous *ADC2*, *SAMDC1*, or *SPMS* shows a preferential induction of stress-related genes [65–67]. When the expression profiles of those Put and Spm overproducer plants are compared, a set of 71 genes always appear to be upregulated and enriched in stress-related genes, including Ca^{2+} signaling-related proteins, as well as several putative transcription factors [66].

The existence of plant PA modulon expression system, in a situation similar to the systems proposed previously in *Escherichia coli* [68] and yeast [69], could be one of the possible explanations for some of the transcriptional changes observed in PA accumulating plants, although the identification of members of this plant PA modulon is still an issue unresolved. In the future, an additional effort in the isolation of the transcription factors controlling expression of genes when PA metabolism is modified is needed to clarify the possible existence of a "PA plant modulon."

Also, most of the transcriptional changes observed could be a consequence of crosstalk of PAs and other signaling routes [70]. There are some evidences of crosstalk between PAs and ABA. Upregulation of PA biosynthetic genes *ADC2*, *SPDS1*, and *SPMS* and accumulation of Put under drought stress in *Arabidopsis* are mainly ABA-dependent responses [34]. Evidence of crosstalk with Spm and ABA is also shown in *SAMDC1* overexpressing *Arabidopsis* plants, with elevated levels of ABA due to the induction of *NCED3*, a key enzyme involved in ABA biosynthesis [67].

A possible link between PAs, Ca^{2+} homeostasis, and stress responses has also been pointed out [56]. Spm control of Ca^{2+} allocation through regulating Ca^{2+} permeable channels, including CAXs, has been described as a possible way of action for the protective role of Spm against high salt and drought stress [13, 71]. Moreover, changes in free Ca^{2+} in the cytoplasm of guard cells are involved in stomatal movement that may explain drought tolerance induced by Spm. What is more, Ca^{2+} signaling genes are one of the gene categories mainly upregulated in Put and Spm overproducer plants [66]. Also, a "Spm signaling pathway" has been proposed to explain the role of enhanced PA accumulation observed during pathogen response in *Arabidopsis* [72, 73]. This signaling pathway could function via the merged signal of Spm-activated Ca^{2+} influx and H_2O_2 produced by Spm degradation for PA oxidases. Both processes are able to trigger mitochondrial dysfunction and activation of the cell death programme [73].

Put, Spd, and Spm also regulate stomatal responses by reducing their aperture and inducing closure [74, 75]. It has been proposed that PAs could regulate stomatal closure in a mechanism involving peroxide production by polyamine oxidation, as well as interactions with nitric oxide (NO) signaling [76]. Thus, PAs could act synergically with reactive oxygen species (ROS) and NO in promoting ABA responses in guard cells [56]. PAs could also regulate stomatal closure responses by their capacity to block fast-activating vacuolar cation channels by their charge properties, as well as affecting protein kinase and phosphatase activities that regulate ion channel functions [56].

In summary, PA action in plant stress responses seems to imply several layers of action. Figure 27.2 illustrates the possible mechanisms underlying enhanced stress tolerance shown by plants with enhanced PA production obtained by transgenic modification. In conclusion, manipulation of polyamine metabolism seems to be a good strategy to obtain tolerant plants, both by the protecting role that PA can exert by their structure and by their capacity to act as a key regulatory molecule in stress responses. Combination of both factors could lead to improved stress tolerance in plants (Figure 27.2).

All these studies demonstrated that a transgenic approach involving PA biosynthetic genes may be a good strategy to improve crop tolerance against harsh environments so as to meet the requirements of a challenging global environment.

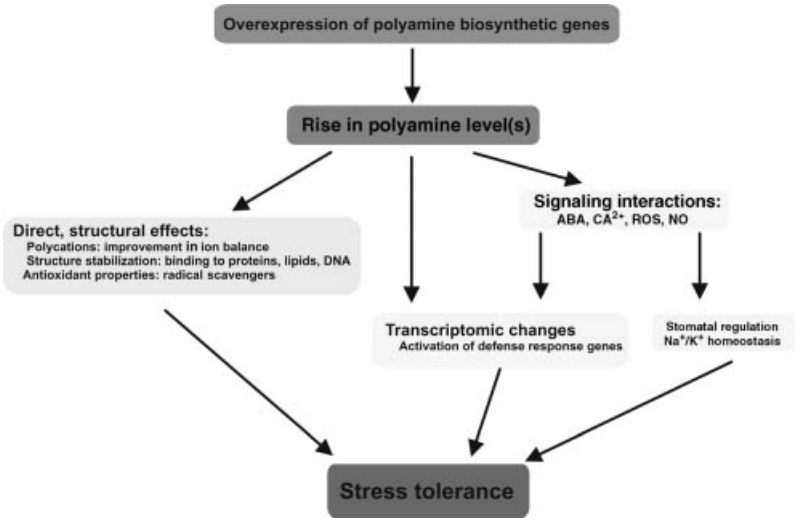

Figure 27.2 Possible mechanisms underlying enhanced stress tolerance via transgenic alteration of polyamine biosynthetic genes. Overexpression of PA biosynthetic genes leads to changes in PA levels in plants (accumulation of individual or total PAs) that could act, on the one hand, as antioxidants to scavenge excessive free radicals or as membrane stabilizers through binding to structures with negatively charged groups, such as DNA, proteins, and lipids, and on the other hand, being able to activate plant defense response mechanisms directly (by unknown mechanisms) and by interactions with other signaling pathways, such as ABA, Ca^{2+}, or NO. Combination of these factors could lead to plant stress tolerance. Adapted from Ref. [33].

Interestingly, broad-spectrum tolerance (salinity, drought, low and high temperature, and parquet toxicity) is observed for some of the transgenic plants overexpressing *ADC*, *SPDS*, or *SAMDC* (Table 27.1). Such multiple abiotic stress tolerance is of practical importance since plants often suffer from several concurrent forms of environmental stress during their life cycle.

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References

1 Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.*, **444**, 139–158.

2 McGloughlin, M.N. (2010) Modifying agricultural crops for improved nutrition. *N. Biotechnol.*, **27**, 494–504.

3 Marais, D.L.D. and Juenger, T.E. (2010) Pleiotropy, plasticity, and the evolution of

plant abiotic stress tolerance. *Ann. N.Y. Acad. Sci.*, **1206**, 56–79.
4 Gill, S.S. and Tuteja, N. (2010) Polyamines and abiotic stress tolerance in plants. *Plant Signal. Behav.*, **5**, 26–33.
5 Alcázar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., Carrasco, P., and Tiburcio, A. (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta*, **231**, 1237–1249.
6 Hamasaki-Katagiri, N., Katagiri, Y., Tabor, C.W., and Tabor, H. (1998) Spermine is not essential for growth of *Saccharomyces cerevisiae*: identification of the SPE4 gene (spermine synthase) and characterization of a spe4 deletion mutant. *Gene*, **210**, 195–201.
7 Imai, A., Matsuyama, T., Hanzawa, Y., Akiyama, T., Tamaoki, M., Saji, H., Shirano, Y., Kato, T., Hayashi, H., Shibata, D., Tabata, S., Komeda, Y., and Takahashi, T. (2004) Spermidine synthase genes are essential for survival of *Arabidopsis*. *Plant Physiol.*, **135**, 1565–1573.
8 Roberts, S.C., Jiang, Y., Jardim, A., Carter, N.S., Heby, O., and Ullman, B. (2001) Genetic analysis of spermidine synthase from *Leishmania donovani*. *Mol. Biochem. Parasitol.*, **115**, 217–226.
9 Urano, K., Hobo, T., and Shinozaki, K. (2005) *Arabidopsis* ADC genes involved in polyamine biosynthesis are essential for seed development. *FEBS Lett.*, **579**, 1557–1564.
10 Imai, A., Akiyama, T., Kato, T., Sato, S., Tabata, S., Yamamoto, K.T., and Takahashi, T. (2004) Spermine is not essential for survival of *Arabidopsis*. *FEBS Lett.*, **556**, 148–152.
11 Minguet, E.G., Vera-Sirera, F., Marina, A., Carbonell, J., and Blazquez, M.A. (2008) Evolutionary diversification in polyamine biosynthesis. *Mol. Biol. Evol.*, **25**, 2119–2128.
12 Wang, X., Ikeguchi, Y., McCloskey, D.E., Nelson, P., and Pegg, A.E. (2004) Spermine synthesis is required for normal viability, growth, and fertility in the mouse. *J. Biol. Chem.*, **279**, 51370–51375.
13 Yamaguchi, K., Takahashi, Y., Berberich, T., Imai, A., Takahashi, T., Michael, A.J., and Kusano, T. (2007) A protective role for the polyamine spermine against drought stress in *Arabidopsis*. *Biochem. Biophys. Res. Commun.*, **352**, 486–490.
14 Alcazar, R., Marco, F., Cuevas, J.C., Patron, M., Ferrando, A., Carrasco, P., Tiburcio, A.F., and Altabella, T. (2006) Involvement of polyamines in plant response to abiotic stress. *Biotechnol. Lett.*, **28**, 1867–1876.
15 Bagni, N. and Tassoni, A. (2001) Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants. *Amino Acids*, **20**, 301–317.
16 Bouchereau, A., Aziz, A., Larher, F., and Martin-Tanguy, J. (1999) Polyamines and environmental challenges: recent development. *Plant Sci.*, **140**, 103–125.
17 Galston, A.W. and Sawhney, R.K. (1990) Polyamines in plant physiology. *Plant Physiol.*, **94**, 406–410.
18 Kumar, A., Taylor, M., Altabella, T., and Tiburcio, A.F. (1997) Recent advances in polyamine research. *Trends Plant Sci.*, **2**, 124–130.
19 Kusano, T., Berberich, T., Tateda, C., and Takahashi, Y. (2008) Polyamines: essential factors for growth and survival. *Planta*, **228**, 367–381.
20 Malmberg, R.L., Watson, M.B., Galloway, G.L., and Yu, W. (1998) Molecular genetic analyses of plant polyamines. *Crit. Rev. Plant Sci.*, **17**, 199–224.
21 Walden, R., Cordeiro, A., and Tiburcio, A.F. (1997) Polyamines: small molecules triggering pathways in plant growth and development. *Plant Physiol.*, **113**, 1009–1013.
22 Richards, F.J., and Coleman, R.G. (1952) Occurrence of putrescine in potassium-deficient barley. *Nature*, **170**, 460–460.
23 Groppa, M.D. and Benavides, M.P. (2008) Polyamines and abiotic stress: recent advances. *Amino Acids*, **34**, 35–45.
24 Chattopadhyay, M.K., Gupta, S., Sengupta, D.N., and Ghosh, B. (1997) Expression of arginine decarboxylase in seedlings of indica rice (*Oryza sativa* L.) cultivars as affected by salinity stress. *Plant Mol. Biol.*, **34**, 477–483.
25 Liu, J.-H., Nada, K., Honda, C., Kitashiba, H., Wen, X.-P., Pang, X.-M., and Moriguchi, T. (2006) Polyamine biosynthesis of apple callus under salt stress: importance of the arginine

decarboxylase pathway in stress response. *J. Exp. Bot*, **57** 2589–2599.

26 Roy, M. and Wu, R. (2002) Overexpression of S-adenosylmethionine decarboxylase gene in rice increases polyamine level and enhances sodium chloride-stress tolerance. *Plant Sci.*, **163**, 987–992.

27 Songstad, D.D., Duncan, D.R., and Widholm, J.M. (1990) Proline and polyamine involvement in chilling tolerance of maize suspension cultures. *J. Exp. Bot.*, **41**, 289–294.

28 Shen, W., Nada, K., and Tachibana, S. (2000) Involvement of polyamines in the chilling tolerance of cucumber cultivars. *Plant Physiol.*, **124**, 431–440.

29 Capell, T., Bassie, L., and Christou, P. (2004) Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. *Proc. Natl. Acad. Sci. USA*, **101**, 9909–9914.

30 Deutsch, A.V., Mitchell, C., Williams, C., Dutt, K., Silvestrov, N., Clement, B., Abukhalaf, I., and Deutsch, D.V. (2005) Polyamines protect against radiation-induced oxidative stress. *Gravit. Space Biol. Bull.*, **1005**, 109–110.

31 Kaur-Sawhney, R., Tiburcio, A.F., Altabella, T., and Galston, A.W. (2003) Polyamines in plants: an overview. *J. Cell Mol. Biol.*, **2**, 1–12.

32 Bell, E. and Malmberg, R.L. (1990) Analysis of a cDNA encoding arginine decarboxylase from oat reveals similarity to the *Escherichia coli* arginine decarboxylase and evidence of protein processing. *Mol. Gen. Genet.*, **224**, 431–436.

33 Liu, J.-H., Kitashiba, H., Wang, J., Ban, Y., and Moriguchi, T. (2007) Polyamines and their ability to provide environmental stress tolerance to plants. *Plant Biotechnol.*, **24**, 117–126.

34 Alcazar, R., Cuevas, J.C., Patron, M., Altabella, T., and Tiburcio, A.F. (2006) Abscisic acid modulates polyamine metabolism under water stress in *Arabidopsis thaliana*. *Physiol. Plant.*, **128**, 448–455.

35 Hao, Y.-J., Kitashiba, H., Honda, C., Nada, K., and Moriguchi, T. (2005) Expression of arginine decarboxylase and ornithine decarboxylase genes in apple cells and stressed shoots. *J. Exp. Bot.*, **56**, 1105–1115.

36 Li, Z. and Chen, S.-Y. (2000) Isolation and characterization of a salt- and drought-inducible gene for S-adenosylmethionine decarboxylase from wheat (*Triticum aestivum* L.). *J. Plant Physiol.*, **156**, 386–393.

37 Cuevas, J.C., Lopez-Cobollo, R., Alcazar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., and Ferrando, A. (2008) Putrescine is involved in *Arabidopsis* freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. *Plant Physiol.*, **148**, 1094–1105.

38 Cuevas, J.C., Lopez-Cobollo, R., Alcazar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., and Ferrando, A. (2009) Putrescine as a signal to modulate the indispensable ABA increase under cold stress. *Plant Signal. Behav.*, **4**, 219–220.

39 Rodríguez-Kessler, M., Alpuche-Solís, A., Ruiz, O.A., and Jiménez-Bremont, J.F. (2006) Effect of salt stress on the regulation of maize (*Zea mays* L.) Genes involved in polyamine biosynthesis. *Plant Growth Regul.*, **48**, 175–185.

40 Urano, K., Yoshiba, Y., Nanjo, T., Igarashi, Y., Seki, M., Sekiguchi, F., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2003) Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. *Plant Cell Environ.*, **26**, 1917–1926.

41 Roy, M. and Wu, R. (2001) Arginine decarboxylase transgene expression and analysis of environmental stress tolerance in transgenic rice. *Plant Sci.*, **160**, 869–875.

42 Altabella, T., Tiburcio, A.F., and Ferrando, A. (2009) Plant with resistance to low temperature and method of production thereof, Spanish patent application WO2010/004070.

43 Alcázar, R., Planas, J., Saxena, T., Zarza, X., Bortolotti, C., Cuevas, J., Bitrian, M., Tiburcio, A.F., and Altabella, T. (2010) Putrescine accumulation confers drought tolerance in transgenic *Arabidopsis* plants over-

expressing the homologous Arginine decarboxylase 2 gene. *Plant Physiol. Biochem.*, **48**, 547–552.
44 Kumria, R. and Rajam, M.V. (2002) Ornithine decarboxylase transgene in tobacco affects polyamines, *in vitro*-morphogenesis and response to salt stress. *J. Plant Physiol.*, **159**, 983–990.
45 Waie, B. and Rajam, M.V. (2003) Effect of increased polyamine biosynthesis on stress responses in transgenic tobacco by introduction of human S-adenosylmethionine gene. *Plant Sci.*, **164**, 727–734.
46 Wi, S., Kim, W., and Park, K. (2006) Overexpression of carnation S -adenosylmethionine decarboxylase gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants. *Plant Cell Rep.*, **25**, 1111–1121.
47 Cheng, L., Zou, Y., Ding, S., Zhang, J., Yu, X., Cao, J., and Lu, G. (2009) Polyamine accumulation in transgenic tomato enhances the tolerance to high temperature stress. *J. Integr. Plant Biol.*, **51**, 489–499.
48 Kasukabe, Y., He, L., Nada, K., Misawa, S., Ihara, I., and Tachibana, S. (2004) Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. *Plant Cell Physiol.*, **45**, 712–722.
49 Kasukabe, Y., He, L., Watakabe, Y., Otani, M., Shimada, T., and Tachibana, S. (2006) Improvement of environmental stress tolerance of sweet potato by introduction of genes for spermidine synthase. *Plant Biotechnol.*, **23**, 75–83.
50 Wen, X.-P., Ban, Y., Inoue, H., Matsuda, N., and Moriguchi, T. (2009) Aluminum tolerance in a spermidine synthase-overexpressing transgenic European pear is correlated with the enhanced level of spermidine via alleviating oxidative status. *Environ. Exp. Bot*, **66**, 471–478.
51 Wi, S. and Park, K. (2002) Antisense expression of carnation cDNA encoding ACC synthase or ACC oxidase enhances polyamine content and abiotic stress tolerance in transgenic tobacco plants. *Mol. Cell*, **13**, 209–220.
52 Hiatt, A. and Malmberg, R.L. (1988) Utilization of putrescine in tobacco cell lines resistant to inhibitors of polyamine synthesis. *Plant Physiol.*, **86**, 441–446.
53 Kasinathan, V. and Wingler, A. (2004) Effect of reduced arginine decarboxylase activity on salt tolerance and on polyamine formation during salt stress in *Arabidopsis thaliana*. *Physiol. Plant.*, **121**, 101–107.
54 Urano, K., Yoshiba, Y., Nanjo, T., Ito, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2004) *Arabidopsis* stress-inducible gene for arginine decarboxylase AtADC2 is required for accumulation of putrescine in salt tolerance. *Biochem. Biophy. Res. Commun.*, **313**, 369–375.
55 Kusano, T., Yamaguchi, K., Berberich, T., and Takahashi, Y. (2007) Advances in polyamine research in 2007. *J. Plant Res.*, **120**, 345–350.
56 Alcázar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., Carrasco, P., and Tiburcio, A.F. (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta*, **231**, 1237–1249.
57 Feuerstein, B.G. and Marton, L.J. (1989) Specificity and binding in polyamine/nucleic acid interactions, in *The Physiology of Polyamines*, vol. **I** (eds U. Bachrach and Y.M. Heimer), CRC Press, Boca Raton, pp. 109–207.
58 Schuber, F. (1989) Influence of polyamines on membrane functions. *Biochem. J.*, **260**, 1–10.
59 He, L., Ban, Y., Inoue, H., Matsuda, N., Liu, J., and Moriguchi, T. (2008) Enhancement of spermidine content and antioxidant capacity in transgenic pear shoots overexpressing apple spermidine synthase in response to salinity and hyperosmosis. *Phytochemistry*, **69**, 2133–2141.
60 Borrell, A., Carbonell, L., Farràs, R., Puig-Parellada, P., and Tiburcio, A.F. (1997) Polyamines inhibit lipid peroxidation in senescing oat leaves. *Physiol. Plant.*, **99**, 385–390.
61 Bors, W., Langebartels, C., Michel, C., and Sandermann, H., Jr. (1989) Polyamines as radical scavengers and protectants against

ozone damage. *Phytochemistry*, **28**, 1589–1595.

62 Kitada, M., Igarashi, K., Hirose, S., and Kitagawa, H. (1979) Inhibition by polyamines of lipid peroxide formation in rat liver microsomes. *Biochem. Biophys. Res. Commun.*, **87**, 388–394.

63 Tadolini, B. (1988) Polyamine inhibition of lipoperoxidation. The influence of polyamines on iron oxidation in the presence of compounds mimicking phospholipid polar heads. *Biochem. J.*, **249**, 33–36.

64 Drolet, G., Dumbroff, E.B., Legge, R.L., and Thompson, J.E. (1986) Radical scavenging properties of polyamines. *Phytochemistry*, **25**, 367–371.

65 Alcazar, R., Garcia-Martinez, J.L., Cuevas, J.C., Tiburcio, A.F., and Altabella, T. (2005) Overexpression of ADC2 in *Arabidopsis* induces dwarfism and late-flowering through GA deficiency. *Plant J.*, **43**, 425–436.

66 Marco, F., Altabella, T., Alcázar, R., Cuevas, J., Bortolotti, C., González, E., Ruiz, O., Tiburcio, A., and Carrasco, P. (2011) Transcriptome analysis of polyamine overproducers reveals activation of plant stress responses and related signalling pathways, in *Omics and Plant Abiotic Stress Tolerance* (ed. N. Tuteja), Bentham Science Publishers, USA.

67 Marco, F., Buso, E., Gruissem, W., Lafuente, T., and Carrasco, P. (2011) A possible role for spermine signalling during abiotic stress, *Plant Physiol.* in press.

68 Igarashi, K. and Kashiwagi, K. (2006) Polyamine modulon in *Escherichia coli*: genes involved in the stimulation of cell growth by polyamines. *J. Biochem.*, **139**, 11–16.

69 Uemura, T., Higashi, K., Takigawa, M., Toida, T., Kashiwagi, K., and Igarashi, K. (2009) Polyamine modulon in yeast – stimulation of COX4 synthesis by spermidine at the level of translation. *Int. J. Biochem. Cell Biol.*, **41**, 2538–2545.

70 Alcázar, R., Planas, J., Saxena, T., Zarza, X., Bortolotti, C., Cuevas, J., Bitrian, M., Tiburcio, A.F., and Altabella, T. (2010) Putrescine accumulation confers drought tolerance in transgenic *Arabidopsis* plants over-expressing the homologous Arginine decarboxylase 2 gene. *Plant Physiol. Biochem.*, **48**, 547–552.

71 Yamaguchi, K., Takahashi, Y., Berberich, T., Imai, A., Miyazaki, A., Takahashi, T., Michael, A.J., and Kusano, T. (2006) The polyamine spermine protects against high salt stress in *Arabidopsis thaliana*. *FEBS Lett.*, **580**, 6783–6788.

72 Mitsuya, Y., Takahashi, Y., Berberich, T., Miyazaki, A., Matsumura, H., Takahashi, H., Terauchi, R., and Kusano, T. (2009) Spermine signaling plays a significant role in the defense response of *Arabidopsis thaliana* to cucumber mosaic virus. *J. Plant Physiol.*, **166**, 626–643.

73 Takahashi, Y., Berberich, T., Miyazaki, A., Seo, S., Ohashi, Y., and Kusano, T. (2003) Spermine signalling in tobacco: activation of mitogen-activated protein kinases by spermine is mediated through mitochondrial dysfunction. *Plant J.*, **36**, 820–829.

74 An, Z., Jing, W., Liu, Y., and Zhang, W. (2008) Hydrogen peroxide generated by copper amine oxidase is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *J. Exp. Bot.*, **59**, 815–825.

75 Liu, K., Fu, H., Bei, Q., and Luan, S. (2000) Inward potassium channel in guard cells as a target for polyamine regulation of stomatal movements. *Plant Physiol.*, **124**, 1315–1326.

76 Yamasaki, H. and Cohen, M.F. (2006) NO signal at the crossroads: polyamine-induced nitric oxide synthesis in plants? *Trends Plant Sci.*, **11**, 522–524.

Part III
Species-Specific Case Studies

Section IIIA Graminoids

Improving Crop Resistance to Abiotic Stress, First Edition.
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28
Wheat: Functional Genomics of Abiotic Stress Tolerance

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Abiotic stresses such as extreme temperatures and water availability, high salt, and deficiencies or toxicity of minerals severely affect productivity of cereal crops worldwide. These stresses become even more disastrous in present environment of climate change. Wheat is one of the most important cereal crops providing approximately 20% percent of calories in human food. Wheat is grown in adverse environments, especially high temperature and low water availability, thereby limiting yield potential. There is ample variation available in abiotic stress tolerance in germplasm of wheat and its wild relatives. However, it has been relatively less exploited due to poor understanding of wheat genome and its molecular basis of stress response. Functional genomics is now widely seen as providing tools for dissecting abiotic stress response in various crop plants. Functional genomics involve many related approaches such as global gene expression profiling, identification of responsive genes/alleles, followed by mutant analysis or transgenic approaches to assign the function of specific gene or its product protein. Since wheat genome is not sequenced, genome-wide collection of ESTs and full-length cDNAs is important for structural and functional analysis of wheat genes responsive to abiotic stresses. This chapter deals with the present knowledge of wheat functional genomics and prospects of molecular breeding for abiotic atress tolerance.

28.1
Introduction

Wheat is an important crop globally and ranks second in production as a cereal crop. Being a staple food, demand for wheat in developing countries is expected to grow at around 2.2% annually similar to the existing rate of production growth; thus, it has been postulated that by 2020 one billion tonnes of wheat would be required to feed the population [1]. Of the various factors influencing wheat crop productivity, abiotic stresses play an important role. Climate change acceleration leading to global warming and higher CO_2 has a major impact on wheat productivity and world wheat production is predicted to decline by as much as 8% from the 2008/09 record volume (FAO, 2009). Thus, to develop improved wheat varieties, molecular genetic

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tools and functional genomics need to be utilized and integrated into the existing breeding practices. Though wheat genome has been refractory due to its large genome size (16 000 Mb), amplification of transposable elements (TEs), coupled with duplication of chromosome segments, and polyploidization, a number of approaches have been developed for wheat genomic research. Development of transgenic wheat, massive EST collections, and cDNA arrays along with comparative genomics approaches have opened new opportunities for wheat improvement. To identify agronomically important genes, the function of genes must be known. Thus, of late, functional genomics is fast emerging as a major tool for wheat improvement.

28.2
Functional Genomics Approaches

28.2.1
Proteomics

For functional analysis of proteins, their structural analysis is necessary. Morita *et al.* [2] developed a novel way of screening protein folding and function by using wheat germ cell-free system. This was the first experimental evidence of the applicability of the wheat germ cell-free protein synthesis system to high-throughput structural analysis of functional proteins. In the same year, Majoul *et al.* [3] analyzed the effect of high-temperature stress on wheat endosperm proteome by employing two-dimensional electrophoresis coupled with analysis by matrix-assisted laser desorption/ionization mass spectrometry and tandem mass spectrometry and characterized heat-induced proteins. The same group in 2004 [4] analyzed the nonprolamin fraction that contains albumins and globulins and functionally identified 42 proteins, some involved in carbohydrate metabolism and others associated with abiotic stresses and heat shock proteins. Using a similar approach, Bahrman *et al.* [5] identified wheat leaf protein expression profile and found that most of them are involved in carbohydrate metabolism. For further enhancing wheat leaf proteome research, Donnelly *et al.* [6] used a combination of protein and expressed sequence tag database and partially characterized the wheat leaf proteome. They found that a majority of proteins were involved in energy production and primary or secondary metabolism. Mamone *et al.* [7] undertook a more detailed characterization of wheat gliadin proteins providing insight into the complex nature of gliadin production. Techniques involving subproteome fractionation combined with two-dimensional electrophoresis and protein identification led to an effective separation of the highly abundant gliadins and glutenins from much less abundant albumins and globulins [8]. An interactome of proteins associated with abiotic stress was developed by using a yeast-two-hybrid GAL4 system and specific protein interactive assays [9]. This revealed a networking of regulatory factors such as phospholipase C and GTP binding protein, VRN1/2, that play vital roles during vernalization, flower initiation, and abscisic acid signaling along with various abiotic stress-related proteins. Gobaa *et al.* [10] using 1BL.1RS translocation lines provided valuable insight into the

endosperm protoeome. They found that in the translocated genotypes the loss of a dimeric α-amylase inhibitor may explain the dough stickiness associated with such genotypes. Upregulation of a certain γ-gliadin with nine cysteine residues indicated the regulation involved in the synthesis of prolamines in the wheat endosperm.

Merlino *et al.* [11] analyzed soluble proteins by two-dimensional electrophoresis and MALDITOF in 112 recombinant inbred lines ("opata 85" × "synthetic W7984") and identified heat shock proteins, β-amylases, UDP-glucose pyrophosphorylases, peroxidases, and thioredoxins. Winning *et al.* [12] examined the implications of different drought treatments on the protein fractions in grains of winter wheat using proton nuclear magnetic resonance spectrosocopy followed by chemometric analysis. The results indicated that protein metabolism is highly influenced by multiple drought events. In a recent study on flooding stress and cell wall proteome of wheat roots, Kong *et al.* [13] employed a gel-based proteomic and LC-MS/MS-based proteomic techniques and found that most upregulated proteins belonged to the category of defense and disease responses. However, downregulation of three proteins such as methionine synthase, β-1,3-glucanases, and β-glucosidase suggested that wheat seedlings respond to flooding stress by restricting cell growth. Thus, the various proteomics studies identified several key proteins leading to a newer discipline called "metabolomics."

28.2.2
Metabolomics

Metabolomics includes a large number of metabolomic reactions that represents the dynamic changes from one condition to the other. Balmer *et al.* [14] analyzed the amyloplast proteome from developing wheat endosperm, and identified 289 proteins involved in carbohydrate metabolism, plastid division, energetics, nitrogen and sulphur metabolism, nucleic acid-related reactions, synthesis of amino acids, isoprenoids, fatty acids, transport, signaling, stress, and related processes. This study thus presents a broad metabolic capability of amyloplasts. On the basis of this proteomic data, Dupont [15] organized the putative amyloplast proteins into proposed metabolic and biosynthetic pathways. An "amyloplast metabolic map" was thus created emphasizing the role played by amyloplasts in endosperm metabolism.

28.2.3
RNA Interference-Based Gene Silencing

RNAi silencing has emerged as a potential tool for functional genomics for hexaploid wheat. It is particularly advantageous for polyploid species such as wheat since it allows the silencing of all homologous gene copies [16]. Yan *et al.* [17] developed the first stable wheat transgenics using RNAi transformation. They reported the reduction of VRN2 RNA by RNA interference resulting in reduced flowering time. In another study by Travella *et al.* [18], RNAi constructs expressing *phytoene desaturase* (PDS) and *ethylene insensitive 2* (EIN2) were introduced by particle bombardment-mediated transformation in wheat and the endogenous target mRNA levels of three homologous genes were seen to decrease due to RNAi silencing. This study also

demonstrated that homozygous transgenic plants have a stronger reduction in target mRNA and thus have severe phenotypic changes compared to heterozygous plants, thus also emphasizing a dosage effect of RNAi in hexaploid wheat. Uauy *et al.* [19] cloned a wild wheat allele encoding an NAC transcription factor (NAM-B1) and showed that reduction in RNA levels of the *TaNAM* genes by RNA interference delayed senescence and decreased protein content, zinc, and iron in wheat grains. Yue *et al.* [20] report the silencing of high molecular weight (HMW) glutenin subunit IDX5 that caused reduction in gluten content and dough quality. Thus, RNAi has been identified as an effective tool to manipulate gene expression for studying functional genomics in polyploids such as wheat.

28.2.4
TILLING

Targeting-induced local lesions in genomes (TILLING) has been reported as an efficient tool for functional genomics in plants [21]. Slade *et al.* [22] demonstrated the use of TILLING, a reverse genetic, nontransgenic approach for the identification of 246 alleles of *waxy* locus encoding granule-bound starch synthase I (GBSSI), in both hexaploid and tetraploid wheat. They also demonstrated that the triple homozygous mutant contains mutations in two *waxy* loci created through TILLING, and a preexisting deletion of the third homologue displayed a near-waxy phenotype. Slade and Knauf [23] reviewed the advantages of TILLING over RNAi transformation, especially for identification of genetic variations in complex genomes required for functional genomic studies. However, till date LI-COR gene analyzers, which use fluorescently labeled primers, were used for TILLING techniques, which is an expensive setup. But recently, a nondenaturing polyacryamide gel setup that uses ethidium bromide was used by Uauy *et al.* [24]. They developed mutant populations of pasta and common wheat and each was characterized by TILLING multiple genes revealing high mutation density in both populations. Thus, TILLING approach provides evidence of the presence of many novel alleles for functional genomic studies.

28.2.5
Transcriptomics

For wheat functional genomics, any alterations in the transcriptome during developmental processes or abiotic stresses would be very useful in assessing gene functions. Wilson *et al.* [25] randomly picked EST clones from 35 cDNA libraries of different stages of wheat grains and plant development and functionally annotated them. High-density microarrays based on these libraries were also produced. In 2005 [26], they presented the use of 9155 wheat unigene cDNA microarrays and reported changes in the wheat embryo transcriptome during maturation and germination. As expected, accumulation of many mRNAs encoding proteins involved in amino acid biosynthesis and metabolism, cell division and cell development, signal transduction, lipid metabolism, energy production, protein turnover,

respiration, initiation of transcription and translation, and ribosomal composition were observed. Also described were changes in abscisic acid signaling and mRNAs encoding *viviparous1* (VP1), suggesting a detailed analysis of transcriptomics to enable manipulation and development of new wheat varieties with superior traits.

Lopato [27] enabled the importance and role of proteins involved in posttranscriptional processes such as splicing in the developing wheat grain. The wheat homologue of *AtRSZ33* splicing factor, designated *TaRSZ38*, was shown to be expressed in the embryo and in mitotically active tissues of the endosperm by *in situ* hybridization and immunodetection. By employing *TaRSZ38* as the bait in a two-hybrid screen, they identified additional proteins that showed high homology to known splicing factors, thus annotating *TaRSZ38* protein to be involved in spliceosome formation. Laudencia-Chingcuanco *et al.* [28] studied global gene expression patterns by using 8 K wheat cDNA microarrays in developing caryopses and functionally annotated genes with respect to their expression in the respective developmental stages. Similarly, Wan *et al.* [29] investigated the transcriptome of wheat developing caryopses by Affymetrix GeneChip® wheat oligonucleotide arrays, which has probes for 55 052 transcripts. Gene expression of 14 550 transcripts was found to be differentially regulated at different development stages.

SAGE (serial analysis of gene expression) for the transcriptome analysis of developing caryopsis in wheat was employed by McIntosh *et al.* [30]. Poole *et al.* [31] generated 71 930 long SAGE tags from 6 libraries of 2 wheat genotypes grown under hot and dry conditions. By using single-copy puroindoline a and b genes, this SAGE analysis revealed the presence of antisense transcripts that may have a role in gene regulation. The regulation by antisense transcripts was further confirmed by Coram *et al.* [32] by developing a novel protocol to assay sense and antisense-strand transcription on the 55 K Affymetrix GeneChip wheat genome array, which is a 3′ *in vitro* transcription (3′IVT) expression array. Of the 110 sense–antisense transcript pairs, most were annotated as genes involved in energy production, suggesting that photosynthesis is likely to be regulated by antisense transcripts. Schreiber *et al.* [33] compared gene expression profiles in barley and wheat by Affymetrix gene chips and reported their transcriptomes to be highly correlated and of use in analyzing functional genomics of these cereals.

28.2.6
Transgenics

There has been an unprecedent increase in our knowledge of functional genomics in wheat by utilizing various newer approaches already discussed. However, the traditional transgenic approach allows the introduction of gene of interest with selective modifications. Enhancing nutritional quality, resisting biotic and abiotic stresses, and herbicide and pesticide resistance would enable to predict the function of introduced genes [34]. There are various reports utilizing wheat as a system for raising transgenics for the characterization of a desired gene. Generating gene knockouts by the introduction of T-DNAs or transposons into wheat has been found to be very useful for analyzing the gene functions [35].

28.3
Wheat Genomic Resources

28.3.1
ESTs

Functional genomics exploits various genomic tools, one of them being EST sequence databases, which has accelerated the pace of gene discovery. There are several publicly available EST databases for wheat where one can download all the available EST data from hexaploid wheat and other Triticeae species. One such site for downloading wheat EST data is http://wheat.pw.usda.gov/NSF/curator/wheat_est.html, where processed 5′ EST data, 5′ EST raw data, quality scores, 3′ EST data, and trace files are available for download. EST contig assembly is performed using a program Phrap (www.phrap.org/). It is necessary to assess the level of library redundancy. Sequencing of individual cDNA libraries was then carried out. The results provide unique ESTs to a specific library. According to the GenBank dbEST database (http://www.ncbi.nih.gov/dbEST/dbEST_summary.html), there are now over 1 067 304 *Triticum aestivum* ESTs available to the public by the ITEC (International Triticeae EST Cooperative) effort and other sequencing projects (dbEST release 012910). Several studies available made use of these EST databases for comparative mapping revealing structural and functional relatedness [35–37]. Several groups worked on construction of cDNA libraries generating ESTs and their utilization for selection of distinct sequence motif unigenes, mapping with wheat aneuploid and deletion stocks, required for wheat genomics and functional genomics studies [38, 39]. Several studies in 2006 focused on the digital expression analysis of these high-quality ESTs obtained from various stages of wheat growth and development and biotic and abiotic stress-related issues such as temperature, drought, photoperiod, moisture, and ABA [40–42]. A large number of stress-responsive genes were identified and their putative functions were analyzed according to gene ontology. Comparison between different wheat genomes (B and D) was also reported on the basis of unique ESTs associated with various abiotic stresses such as heat, cold, drought, salinity, and aluminum by Ramalingam *et al.* [43].

28.3.2
Full-Length cDNA Resource

Construction of full-length cDNAs provides the putative annotation based on homology search against several protein databases. One database that provides full-length coding sequences for wheat along with their annotation is TriFLDB: Triticeae full-length CDS Data Base (http://trifldb.psc.riken.jp/index.pI). The current version provides 8530 putative full-length wheat cDNA sequence and their annotations. Similarly, another wheat genetic resource database (KOMUGI) provides several tissue-specific, biotic and abiotic stress-related cDNA sequences (www.shigen.nig.ac.jp/wheat/komugi/ests/tissue). cDNA resource/library has also been constructed from young spikelets of hexaploid wheat and 24056 ESTs were obtained from both ends of cDNA clones [44]. Till date, it has been found useful in mainly

biotic stresses. There are studies describing the construction of full-length cDNA library from wheat stripe rust fungus [45]. Kawaura *et al.* [46] generated a high-quality, full-length cDNA resource for common wheat and 6162 clones were sequenced. About 10% of the clones were uniquely present in wheat while rest showed high homology to those of rice. They analyzed their expression patterns in 28 different tissues and abiotic stress treatments, the differential expression suggests that molecular selection occurred during the diversification of wheat and rice and is considered a valuable asset for functional genomics in wheat.

28.3.3
Wheat Mutants

As a wheat genetics resource, reverse genetics approach includes the creation of mutants by targeting specific gene to assess its function. A collection of several knockout mutants in wheat has been generated to assess the function of genes involved in biotic stress. Several mutants were isolated in wheat that showed increased resistance toward fungal causal agent of yellow rust, brown rust, blotch fungus such as *Mycosphaerella graminicola* and *Pyrenophora tritici-repentis* [47–51]. For understanding fungal gene expression related to pathogenicity, Goswami *et al.* [52] constructed 3 cDNA libraries and the probable functions of 49 genes inferred. One mutant showed reduced sporulation and delayed spread of *Fusarium* on wheat. Several studies also focused on the identification and molecular characterization of *waxy* mutants in wheat [53–55]. Three WAXY proteins (granule-bound starch synthase I) have been characterized for the development of new waxy wheat lines.

Many vernalization-related mutants such as VRN1 and VRN2, the *mvp* mutants (maintained vegetative phase) of einkorn wheat (*T. monococcum*) that has *null* alleles of VRN1, have also been very useful in analyzing genes that control photoperiod and flowering-time genes [56–59]. The analysis indicated a genetic network of flowering-time genes in wheat leaves and the need for a more detailed molecular characterization of the *mvp* mutants. However, attention is now focused on creation of mutants with respect to abiotic stress. Thermotolerant mutants [60] and salt stress mutants [61] have already been characterized to study the genetic basis of stress tolerance in wheat.

28.3.4
Introgression Lines

Introgression lines derived from multiple inbred strains serve as a powerful resource for studying multiple quantitative trait loci by introducing specific traits. For the generation of biotic/abiotic stress-resistant wheat, development of alien addition/substitution lines, and thus introgression lines, become essential. However, the pairing homologous 1 (Ph1) locus restricts chromosome pairing and recombination during meiosis. Thus, to uncover the mechanism of the Ph1 locus, various Ph1 and Ph2 mutants and introgression lines have been developed [62–64]. Several young isogenic lines and their genetic analyses have also contributed to the detailed characterization of regulatory regions of VRN1 vernalization genes [65, 66]. For

understanding the mechanism of resistance for biotic stress tolerance, near-isogenic lines for wheat rust resistance gene Lr34/Yr18 were examined [67]. For abiotic stress, several RIL (recombinant inbred line) and NIL (near-isogenic line) populations were analyzed for the effect of heat stress on CMS (cellular membrane thermostability) and GluD-1 [68, 69]. To understand the role of each chromosome during cold tolerance, 20 isogenic lines of wheat were analyzed [70]. However, further studies are essential for understanding the mechanism of resistance for biotic and abiotic stress tolerance.

28.4 Wheat Functional Genomics for Various Stresses

28.4.1 Drought Stress

Among several abiotic stresses, water stress is the most widespread in wheat. Several studies therefore focus on the accumulation of LEA (late embryogenesis abundant) proteins, osmolytes (proline, glycine, etc.), as adaptive mechanisms for protection against drought stress. Pellegrineschi *et al.* [71] expressed the *AtDREB* gene (dehydration responsive element binding) under the control of a stress-induced promoter *rd29* in wheat. The transgenics raised showed some tolerance to drought stress by a 10 day delay in wilting. Selote *et al.* [72] demonstrated limited ROS (reactive oxygen species) accumulation in the leaves and roots of drought-acclimated wheat (*T. aestivum* cv.306) that caused minimal membrane damage. In another related study, Xu *et al.* [73] examined the transcript accumulation of wheat PP2Ac-1 (catalytic subunit of protein phosphatase 2A) during water stress. Transgenic tobacco plants overexpressing *TaPP2Ac-1* gene were found to be resistant to water deficit. A "feedback mechanism" was proposed to be operating under drought stress conditions in mitochondria involving ROS production by Pastore *et al.* [74]. The function of wheat calreticulin (*TaCRT)* toward drought stress was demonstrated by *TaCRT*-overexressing tobacco (*Nicotiana benthamiana*) plants that showed enhanced drought resistance by higher WUE (water use efficiency), WRA (water retention ability), RWC (relative water content), and lower MDR (membrane damaging ratio) under water deficit conditions [75]. Transcriptome profile of wheat "opata" roots under drought stress revealed 394 transcripts differentially regulated with a fold change of at least 1.5 between stressed and control roots [76]. The genes of importance were putative glucanases and class III peroxidases. Kam *et al.* [77] identified 47 Q-type zinc finger protein genes from *T. aestivum* and analyzed their expression profile in different organs during leaf development and aging, drought stress, and ABA and sucrose treatment. They concluded that 30 genes were predominantly expressed in roots and 37 *TaZFP* genes responded to drought stress. Recently, Ergen *et al.* [78] compared global expression profiles of drought-tolerant and -sensitive wild emmer wheat genotypes at two different time points in two different tissues (root and leaf) using the Affymetrix GeneChip wheat genome array. The data revealed several unique genes and signal related pathways such as IP3, ethylene, ABA-dependent signaling,

and a faster induction of ABA-dependent transcription factors in the tolerant genotype, suggesting some unique transcriptome pathways in wild emmer wheat that correlated with their ability to withstand drought conditions.

28.4.2 Salinity Stress

Wheat is exposed to salt stress as it is mainly grown under irrigated and rain-fed conditions [79]. Na^+ exclusion, Na^+ compartmentalization, and K^+ uptake are some of the adaptive mechanisms reported in wheat. Colmer *et al.* [80] discussed different sources of Na^+ exclusion among various genomes that make up tetraploid wheat (*T. durum*), hexaploid bread wheat (*T. aestivum*), and wild relatives such as *Aegilops* spp. and *Thinopyrum* spp. In the same year, Kawaura *et al.* [81] constructed a 22 K wheat oligo-DNA microarray with 148 676 ESTs of common wheat. They analyzed 1811 genes whose expression changed more than twofold in response to salt stress. Such global gene profiling studies help to understand the mechanism of salt tolerance in wheat.

Using a comparative genomics approach, Huang *et al.* [82] utilized rice genome sequence and wheat EST data to identify and characterize a candidate gene for *Nax1* responsible for low Na^+ concentration in leaf blades. They reported two putative sodium transporter genes (*TmHKT7A1–2*) related to *OsHKT7*, out of which *TmHKT7A2* was expressed in roots and leaf sheaths of salt-tolerant durum wheat line 149, which correlated well with the physiological role of *Nax1* in reducing Na^+ concentration.

Another adaptive measure other than Na^+ exclusion studied in wheat is its ability to retain K^+. Cuin *et al.* [83] used the noninvasive ion flux measuring technique to measure K^+ flux from roots in two bread and two durum wheat genotypes, contrasting in their salt tolerance. Kawaura *et al.* [84] designed oligo-DNA microarrays from approximately 32 000 unique wheat genes. They observed 5996 genes differentially expressed when treated with salt for different intervals by more than twofold. These genes were assigned functions using gene ontology (GO) and categorized as transcription factors, transcriptional regulators, DNA binding functions, and some as early-responsive genes and late-responsive genes as transferase and transporters. For creating novel germplasm for improving salt tolerance in bread wheat, Mullan *et al.* [85] chose *Lophopyrum elongatum* (tall wheat grass). They induced recombination of chromosome 3E from tall wheat grass with chromosome 3A and 3D from bread wheat and using molecular marker analysis and genomic *in situ* hybridizations provided a novel germplasm that could be deployed to enhance Na^+ exclusion in bread wheat, thus providing novel functional genes that also give an insight into the mechanism of salt tolerance in wheat.

28.4.3 Low-Temperature Stress

Low-temperature stress/cold stress to wheat plants produces many morphological, biochemical, and physiological changes. Thus, expression profile of differentially

expressed genes under cold stress is desirable and advantageous. For the same purpose, Gulick *et al.* [86] performed microarray analysis with cDNA inserts from 1184 wheat ESTs that represented 947 genes. Transcriptome comparison of winter and spring wheat revealed more than 300 genes expressed under cold stress of which 65 were differentially regulated between the cultivars. To study the function of wheat alternative oxidase (*AOX*) genes under low temperature, Sugie *et al.* [87] produced transgenic plants by introducing wheat *aox1a* under the control of CaMV 35 S promoter in *Arabidopsis thaliana*. The results revealed that levels of reactive oxygen species decreased in transgenic plants under low-temperature stress and recovery of total respiration activity under low temperature occurred rapidly in the transgenic plants, suggesting that *AOX* alleviates oxidative stress when the cytochrome pathway of respiration is inhibited under abiotic conditions.

Monroy *et al.* [88] compared the gene expression in winter wheat cultivar CDC Clair and spring wheat cultivar Quantum, under cold stress by a 5740 feature cDNA amplicon microarray that was enriched for signaling and regulatory genes. About 450 genes were found to be regulated by cold and among them about 130 were for signaling or regulatory gene candidates that included various transcription factors, protein kinases, ubiquitin ligases, and GTP, RNA, and calcium binding proteins. Kobayashi *et al.* [89] isolated *Wlip19*; wheat lip19 homologue that encoded a *bZIP-type* transcription factor, which expressed under low temperature in seedlings and was found in higher amount in the freezing-tolerant cultivar. Transgenic tobacco plants expressing *Wlip19* showed enhanced tolerance to freezing and other abiotic stress as well. Under the control of promoter sequences of four wheat *Cor/Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18*, and *Wrab19*, expression of a GUS reporter gene was enhanced by *Wlip19* expression in wheat callus and tobacco plants. Thus, it was concluded that WLIP19 acts as a transcription regulator of *Cor/Lea* genes under abiotic stress tolerance.

28.4.4
High-Temperature Stress

The effect of global warming on various crop plants can be estimated by the yield loss, which is maximum in the case of wheat [90]. However, not many studies have been reported detailing the expression analysis or assessing the function of genes that can contribute to functional genomics in case of wheat under heat stress. Nonetheless, Gallie *et al.* [91] analyzed the translation elongation factors from wheat during heat shock treatment. They examined the protein levels and isoelectric state of elongation factor (EF) 1α and 2 in the regulation of translation. EF2 expression level from wheat seeds decreased slightly under heat stress; however, no changes were observed in the number or levels of isoforms observed. Campbell *et al.* [92] cloned two heat stress-inducible members of HSP101/ClpB family (*TaHSP101 B* and *TaHSP101 C*) that were found to be inducible by heat stress treatments, dehydration, and ABA, thus suggesting role of *HSP101* in osmotic stress responses. Gulli *et al.* [93] isolated and characterized four cDNAs encoding HSP101 in durum wheat. The expression analysis revealed their differential regulation under heat stress. Thus, this data would be useful in analyzing the functions of *HSP101* family members.

Genome-wide gene expression profiling using GeneChip wheat genome array of two wheat genotypes, Chinese Spring (CS), susceptible to heat stress, and "TAM107," tolerant to heat stress, was undertaken by Qin *et al.* [94] and found 6560 probe sets responsive to heat treatment. These genes belong to heat shock proteins (HSPs), transcription factors, phytohormone biosynthesis/signaling, calcium and sugar signal pathways, RNA metabolism, ribosomal proteins, primary and secondary metabolism, and biotic and abiotic stresses. Ristic *et al.* [95] observed *rubisco activase* (*RCA*) expression in four genotypes of wheat and a positive correlation was found among the wheat 45–46 kDa *RCA* of different genotypes under heat stress, suggesting an important role played by endogenous levels of *RCA* as well.

Detailed transcriptome analysis has been carried out through suppression subtractive hybridization of heat-stressed and nonstressed tissues of wheat at three different development stages, that is, young seedlings, prepollinated flowers, and developing grains [96]. In all, 5500 ESTs were generated, out of which 3516 high-quality ESTs were submitted to GenBank. Their differential expression was confirmed by cDNA macroarray and by Northern/RT-PCR analysis. Some of the transcripts that showed high induction by high-temperature stress are stress-related proteins. Real-time PCR of selected genes gave further insight into their putative roles that, however, needs functional validation through transgenic technology.

28.4.5
Signaling Network

Plants respond to various abiotic stresses through their interconnection, which contributes to protection of the plant against unfavorable environmental conditions. There are various mechanisms by which plants respond to abiotic stresses such as drought, salt, and cold stress. However, Ca^{2+} signaling is a universal early-responsive mechanism that involves calcium sensors, such as calmodulin, calmodulin-like proteins, calcineurin B-like proteins, and calcium-dependent protein kinases [97]. Wang *et al.* [98] functionally analyzed a novel Ca^{2+}-permeable channel gene *TaTPC1* from wheat. This putative membrane protein showed enhanced expression under high salinity, PEG, low temperature, and ABA. *TaTPC1*-overexpressing transgenic plants exhibit more stomatal closing under Ca^{2+} than the control plants, suggesting a role for the calcium channels in response to various abiotic stresses in wheat. Charron *et al.* [99] identified and characterized plant lipocalins and lipocalin-like proteins. They analyzed the expression of wheat lipocalins under various abiotic stress responses such as PEG-induced dehydration, salt, high-temperature, and freezing stress that correlated with the capacity of the plant to develop freezing tolerance. Data mining also revealed that lipocalins are present in desiccation-tolerant red algae *Porphyra yezoensis* and the cryotolerant yeast *Debaryomyces hansenii*, suggesting the putative function as protection of the photosynthetic system against temperature stress.

Egawa *et al.* [100] isolated a DREB2 homologue *Wdreb2*, a candidate gene for a transcription factor of the *Cor/Lea* genes; its detailed expression analysis indicated its activation in drought, cold, salt, and exogenous ABA treatment. They also showed

three transcript forms of *Wdreb2* (α, β, and γ) through alternative splicing and their differential expression. Faik *et al.* [101] reported the characterization of 34 wheat fasciclin-like arabinogalactan-proteins (FLAs) by expression and bioinformatics analysis. The wheat EST databases and RNA gel blots indicated that most of the *TaFLA* genes are expressed in reproductive organs and roots and two of them are upregulated by cold treatment in roots. This study laid the foundation for analysis of the function of each *TaFLA* protein in plant development during abiotic stress responses. Stephenson *et al.* [102] identified 37 *NY-F* genes in wheat in the global DNA databases. NF-Y is a trimeric complex that binds to the CCAAT-box, highly conserved promoter element in eukaryotes. Quantitative RT-PCR revealed that some of them were predominantly expressed in the endosperm and three were activated under drought conditions, indicating a plant-specific biological role for this transcription factor family.

Xu *et al.* [103], for the first time, isolated a *TaERF1* (*T. aestivum* ethylene responsive factor 1) with a conserved DNA binding domain, a conserved N-terminal motif (MCGGAIL), and a putative phosphorylation site (TPDITS) in the C-terminal region, substrate for *TaMAPK1* protein kinase. Transactivation assays of *TaERF1* in tobacco leaves revealed the activation of GUS reporter gene driven by GCC-box indicating that *TaERF1* binds to the GCC-box and transactivates reporter gene expression. Expression analysis of *TaERF1* revealed its induction in drought, salinity, low-temperature stress, and exogenous ABA, ethylene, and salicylic acid indicating its involvement in multiple stress and signal transduction pathways. Expectedly, overexpression of the *TaERF1* gene improved tolerance to abiotic stresses in transgenic plants. A study by Brini *et al.* [104] has shown that transgenic *A. thaliana* plants overexpressing one of the two wheat vacuolar Na^+/H^+ antiporter *TNHX1* or H^+ pyrophosphatase *TVP1* showed resistance to high salt concentration and water deficit conditions. There is increased osmotic adjustment in transgenic plants due to accumulation of more Na^+ and K^+ in their leaf tissue than the control plants. Similarly, Kobayashi *et al.* [105] generated tobacco transgenic plants expressing *Wdreb2* (wheat DREB2 homologue) with clearly improved freezing and osmotic stress tolerance in tobacco plants. The GUS expression was enhanced by cold, drought, and ABA treatment under the control of *Cor/Lea* promoter sequences in transgenic plants, indicating WDREB2 could be a transcription factor that positively regulates wheat *Cor/Lea genes* under various abiotic stresses. Jung *et al.* [106] examined four wheat *O*-methyl transferases (OMT) genes, involved in primary and secondary metabolism. Their tissue-specific expression and differential regulation in response to various abiotic stresses and hormones such as PEG, Cold, NaCl, UV-B, wounding, and methyl jasmonate, salicylic acid, ethylene, and ABA was studied that signifies functional diversity of wheat *OMT*s in response to differential expression.

Three homologues of the DBF (dehydration-resposive element binding factors) gene family in wheat were isolated by Xu *et al.* [107] and designated as *TaAIDFs a,b,c* (*T. aestivum* abiotic stress-induced DBFs). *TaAIDFa* transcript was upregulated under drought, salinity, cold stress, and exogenous ABA. Also, overexpression of *TaAIDFa* in transgenic *Arabidopsis* showed enhanced tolerance toward drought and osmotic stresses. Shaw *et al.* [108] identified 31 *Dof* (DNA binding with one finger) genes in

bread wheat and studied their expression analysis. While most of the *TaDof* genes expressed in vegetative organs, they were also downregulated by drought and activated by light and dark cycle. The data indicate the number of genes involved in photosynthetic process or sucrose transport, suggesting its potential role in the photosynthetic process. Kovacs *et al*. [109] compared the effects of cold, osmotic stress, and ABA on polyamine accumulation in wheat variety CS and in two-derived chromosome 5A substitution lines (*T. spelta* 5A) and CS (Cheyenne 5A) with lower and higher levels of freezing tolerance, respectively. The differential regulation provides an insight into the involvement of polyamines in abiotic stress adaptation to plants and the possible regulatory role of chromosome 5A. Mao *et al*. [110] generated transgenic *Arabidopsis* plants overexpressing wheat *TaSnRK2.4* (sucrose nonfermenting 1-related protein kinase 2) under the control of CaMV35S promoter resulting in enhanced tolerance to drought, salt, and freezing stresses. Morphological and physiological assays revealed decreased rate of water loss, enhanced higher relative water content, strengthened cell membrane stability, improved photosynthesis and increased osmotic potential, indicating *TaSnRK2.4* acts as a multifunctional regulatory factor in *Arabidopsis*.

A comprehensive study of CDPK was provided by Li *et al*. [111]. They isolated 14 full-length cDNA sequences of CDPKs and analyzed their expression profile under various biotic and abiotic stresses such as cold, H_2O_2, salt, drought, powdery mildew, abscisic acid, gibberellic acid, suggesting their role in multiple signal transduction pathways. Using knowledge of this crosstalk between biotic and abiotic stress signaling pathways involving CDPKs, Li *et al*. [112] developed a model depicting possible roles of wheat CDPK genes under various biotic and abiotic stress conditions. Thus, utilizing this knowledge of CDPK genes and other Ca^{2+} sensors provides a strong foundation for further functional characterization of genes involved in Ca^{2+} signaling-mediated stress tolerance in wheat plants.

28.5
Wheat Functional Genomics for Plant Growth and Development

Berna *et al*. [113] studied the expression of germin-like proteins, which constitute a ubiquitous family of plant proteins. They observed the expression of *gf-2.8* gene in abiotic stresses such as heavy metal ions Cd^{2+}, Cu^{2+}, and Co^{2+}, polyamines, and biotic stress such as wounding and TMV infection, thus suggesting its role in several aspects of plant growth and development. Yao *et al*. [114] identified 18 *expansin* genes from wheat that were expressed in leaf, root, and the developing seed. Four *β-expansin* genes were expressed in the internode tissue in F1 hybrids, suggesting important roles of *expansin* gene family in growth and development. Kulshreshtha *et al*. [115] isolated and functionally characterized a *PHY*gene (*TaPHYC*) from wheat that shared structural similarity with rice *PHYC* containing four exons and three introns. Reverse transcriptase-PCR (RT-PCR) analysis showed it to be a constitutively expressed gene in all organs under light/dark conditions, but showed maximum upregulation in 3 day-old dark-grown seedlings.

Zhao *et al*. [116] isolated 42 putative wheat MADS-box genes and their expression analysis revealed differential expression patterns in various organs and development stages such as primary root tips, whole spikelets (lodicules, palea, stamens, and pistil), leaf, and stem indicating their universal role in wheat growth and development. Paolacci *et al*. [117] functionally characterized 45 MIKC-type MADS-box genes by RT-PCR and northern hybridization revealing the putative functions of the genes by comparing expression patterns with functionally characterized *Arabidopsis* MADS-box genes. Recently, Kovalchuk *et al*. [118] isolated and characterized *TaPR60* gene from bread wheat, which encodes a small cysteine-rich protein with a hydrophobic signal peptide that might direct the TaPR60 protein to a secretory pathway. To understand the function of this gene, yeast two-hybrid screen of a cDNA library prepared from developing wheat grain was performed, where TaPR60 was used as a bait and the interacting proteins were found to be involved in the proteolytic processing and secretion of *TaPR60*.

28.6 Comparative Genomics

Global comparative sequence analysis at the level of both DNA and protein sequence is performed with the aim of deriving structural, functional, and evolutionary relationships across several species [119]. *Arabidopsis* and rice are considered two model species suitable for comparative genomics. *Arabidopsis* is used as a model for all flowering plants, while rice is used as a model for genomes of cereals like wheat. Through comparative analysis, Mullan *et al*. [120] studied genes that control Na^+ accumulation, such as *HKT1* and *SOS1* in *Arabidopsis*; wheat orthologues were identified, characterized, and confirmed through similar intron–exon structure in *Arabidopsis* and rice. On the basis of additional exons identified in the predicted *NHX1* and *SOS1* genes of rice and wheat compared to *Arabidopsis*, they suggested evolutionary relationships among them. Boutrot *et al*. [121] performed comparative analysis of rice nonspecific lipid transfer protein (nsLTPs) and wheat ESTs and thus identified 156 putative wheat *LTPs*, where the majority (91) were from "Chinese Spring" cultivar. Thus, plant *nsLTPs* were categorized on the basis of sequence similarity and/or phylogenetic clustering.

Recently, *Brachypodium distachyon* (L.) is emerging as a model system for cereals because of its small genome, short life cycle, self-fertility, diploid accessions, and simple growth requirements [122]. It is phylogenetically very similar to wheat and barley, and thus various genomic resource studies involving the construction of cDNA libraries, BAC libraries, EST sequences, linkage map, and the complete genome sequence are under development [123]. This group has also developed a transformation method with efficiency as high as 41%, which will play an important role in comparative genomics. Microcolinearity was found to be more conserved for the Q gene region between wheat and rice than between wheat and *Brachypodium*, but phylogenetic analysis indicates *Brachypodium* is more closely related to wheat than rice [124]. Its syntenic relationship with rice and wheat is being analyzed by

structural characterization of its genome and construction of a BAC-based physical map [125, 126].

28.7 Conclusions

With utilization of wheat genomic resources along with functional genomics approaches, though slow yet a steady progress in wheat functional genomics is evident. Since complete wheat genome sequencing is not possible in near future, *Brachypodium* genome would serve as a platform for identification and functional analysis of genes of importance in wheat. In the meantime, availability of more ESTs/full-length cDNAs approach would be used for allele mining and molecular breeding. With the availability of cereal genomes such as rice, maize, sorghum, and so on, the field of comparative genomics appears promising and complex genomes such as wheat may also benefit with the progress in the area of plant genomics. It is expected that the coming century will witness landmark discoveries and pathfinding leads in our understanding of plant biology in general and help plant improvement in unprecedented ways.

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References

1 Braun, H.J., Payne, T.S., Morgounov, A.I. *et al.* (1998) The challenge: one billion tons of wheat by 2020, in *Saskatchewan* (ed. A.E. Slinkard), University Extension Press, Canada.
2 Morita, E.H., Sawasaki, T., Tanaka, R. *et al.* (2003) *Protein Sci.*, **12**, 1216–1221.
3 Majoul, T., Bancel, E., Triboï, E. *et al.* (2003) *Proteomics*, **3**, 175–183.
4 Majoul, T., Bancel, E., Triboi, E. *et al.* (2004) *Proteomics*, **4**, 505–513.
5 Bahrman, N., Negroni, L., Jaminon, O., and Le Gouis, J. (2004) *Proteomics*, **4**, 2672–2684.
6 Donnelly, B.E., Madden, R.D., Ayoubi, P. *et al.* (2005) *Proteomics*, **5**, 1624–1633.
7 Mamone, G., Addeo, F., Chianese, L. *et al.* (2005) *Proteomics*, **5**, 2859–2865.
8 Hurkman, W.J. and Tanaka, C.K. (2007) *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **849**, 344–350.
9 Tardif, G., Kane, N.A., Adam, H. *et al.* (2007) *Plant Mol. Biol.*, **63**, 703–718.
10 Gobaa, S., Bancel, E., Kleijer, G. *et al.* (2007) *Proteomics*, **7**, 4349–4357.
11 Merlino, M., Leroy, P., Chambon, C. *et al.* (2009) *Theor. Appl. Genet.*, **118**, 1321–1337.
12 Winning, H., Viereck, N., Wollenweber, B. *et al.* (2009) *J. Exp. Bot.*, **60**, 291–300.
13 Kong, F.J., Oyanagi, A., and Komatsu, S. (2010) *Biochim. Biophys. Acta*, **1804**, 124–136.
14 Balmer, Y., Vensel, W.H., DuPont, F.M. *et al.* (2006) *J. Exp. Bot.*, **57**, 1591–1602.
15 Dupont, F.M. (2008) *BMC Plant Biol.*, **8**.

16 Lawrence, R.J. and Pikaard, C.S. (2003) *Plant J.*, **36**, 114–121.
17 Yan, L., Loukoianov, A., Blechl, A. *et al.* (2004) *Science*, **303**, 1640–1644.
18 Travella, S., Klimm, T.E., and Keller, B. (2006) *Plant Physiol.*, **142**, 6–20.
19 Uauy, C., Distelfeld, A., Fahima, T. *et al.* (2006) *Science*, **314**, 1298–1301.
20 Yue, S.J., Li, H., Li, Y.W. *et al.* (2007) *J. Cereal Sci.*, **47**, 153–161.
21 Mccallum, C.M., Comai, L., Greene, E.A., and Henikoff, S. (2000) *Plant Physiol.*, **123**, 439–442.
22 Slade, A., Fuerstenberg, S.I., Loeffler, D. *et al.* (2005) *Nat. Biotech.*, **23**, 75–81.
23 Slade, A.J. and Knauf, V.C. (2005) *Trans. Res.*, **14**, 109–115.
24 Uauy, C., Paraiso, F., Colasuonno, P. *et al.* (2009) *BMC Plant Biol.*, **9**, 115.
25 Wilson, I.D., Barker, G.L., Beswick, R.W. *et al.* (2004) *Plant Biotechnol. J.*, **2**, 495–506.
26 Wilson, I.D., Barker, G.L.A., Lu, C. *et al.* (2005) *Funct. Integr. Genomics*, **5**, 144–154.
27 Lopato, S., Borisjuk, L., Milligan, A.S. *et al.* (2006) *Plant Mol. Biol.*, **62**, 637–653.
28 Laudencia-Chingcuanco, D.L., Stamova, B.S., You, F.M. *et al.* (2007) *Plant Mol. Biol.*, **63**, 651–668.
29 Wan, Y., Poole, R.L., Huttly, A.K. *et al.* (2008) *BMC Genomics*, **9**, 121.
30 McIntosh, S., Watson, L., Bundock, P. *et al.* (2007) *Plant Biotech. J*, **5**, 69–83.
31 Poole, R.L., Barker, G.L.A., Werner, K. *et al.* (2008) *BMC Genomics*, **9**, 475.
32 Coram, T.E., Settles, M.L., and Chen, X. (2009) *BMC Genomics*, **10**, 253.
33 Schreiber, A.W., Sutton, T., Caldo, R.A. *et al.* (2009) *BMC Genomics*, **10**, 285.
34 Khurana, P., Chauhan, H., and Desai, S.A. (2008) *Compendium of Transgenic Crop Plants: Transgenic Cereals and Forage Grasses* (eds C. Kole and T.C. Hall), Blackwell Publishing Ltd, pp. 83–100.
35 Kantety F R.V., La Rota, M., Matthews, D.E., and Sorrells, M.E. (2002) *Plant Mol. Biol.*, **48**, 501–510.
36 Clarke, B., Lambrecht, M., and Rhee, S.Y. (2003) *Funct. Integr. Genomics*, **3**, 33–38.
37 Hattori, J., Ouellet, T., and Tinker, N.A. (2005) *Genome*, **48**, 197–206.
38 Zhang, D., Choi, D.W., Wanamaker, S. *et al.* (2004) *Genetics*, **168**, 595–608.
39 Lazo, G.R., Chao, S., Hummel, D.D. *et al.* (2004) *Genetics*, **168**, 585–593.
40 Houde, M., Belcaid, M., Ouellet, F. *et al.* (2006) *BMC Genomics*, **7**, 149.
41 Chao, S., Lazo, G.R., You, F. *et al.* (2006) *Genome*, **49**, 531–544.
42 Mochida, K., Kawaura, K., Shimosaka, E. *et al.* (2006) *Mol. Genet. Genomics*, **276**, 304–312.
43 Ramalingam, J., Pathan, M.S., Feril, O. *et al.* (2006) *Genome*, **49**, 1324–1340.
44 Ogihara, Y., Mochida, K., Kawaura, K. *et al.* (2004) *Genes Genet. Sys.*, **79**, 227–232.
45 Ling, P., Wang, M., Chen, X., and Campbell, K.G. (2007) *BMC Genomics*, **8**, 145.
46 Kawaura, K., Mochida, K., Enju, A. *et al.* (2009) *BMC Genomics*, **10**, 271.
47 Koebner, R. and Hadfield, J. (2001) *Genome*, **44**, 45–49.
48 Friesen, T.L., Rasmussen, J.B., Kwon, C.Y. *et al.* (2002) *Phytopathology*, **92**, 38–42.
49 Boyd, L.A., Smith, P.H., Wilson, A.H., and Minchin, P.N. (2002) *Genome*, **45**, 1035–1040.
50 Adachi, K., Nelson, G.H., Peoples, K.A. *et al.* (2002) *Curr. Genet.*, **42**, 123–127.
51 Smith, P.H., Howie, J.A., Worland, A.J. *et al.* (2004) *Mol. Plant Microbe Interact.*, **17**, 1242–1249.
52 Goswami, R.S., Xu, J.R., Trail, F. *et al.* (2006) *Microbiology*, **152**, 1877–1890.
53 Saito, M., Konda, M., Vrinten, P. *et al.* (2004) *Theor. Appl. Genet.*, **108**, 1205–1211.
54 Saito, M. and Nakamura, T. (2005) *Theor. Appl. Genet.*, **110**, 276–282.
55 Monari, A.M., Simeone, M.C., Urbano, M. *et al.* (2005) *Theor. Appl. Genet.*, **110**, 1481–1489.
56 Dubcovsky, J., Loukoianov, A., Fu, D. *et al.* (2006) *Plant Mol. Biol.*, **60**, 469–480.
57 Shitsukawa, N., Ikari, C., Shimada, S. *et al.* (2007) *Genes Genet. Syst.*, **82**, 167–170.
58 Shimada, S., Ogawa, T., Kitagawa, S. *et al.* (2009) *Plant J*, **58**, 668–681.
59 Distelfeld, A. and Dubcovsky, J. (2010) *Mol. Genet. Genomics*, **283**, 223–232.
60 Mullarkey, M. and Jones, P. (2000) *J. Exp. Bot.*, **51**, 139–146.
61 Huo, C.M., Zhao, B.C., Ge, R.C. *et al.* (2004) *Yi Chuan Xue Bao*, **31**, 408–414.

62 Al-Kaff, N., Knight, E., Bertin, I. *et al.* (2008) *Ann. Bot.*, **101**, 863–872.
63 Sidhu, G.K., Rustgi, S., Shafqat, M.N. *et al.* (2008) *Proc. Natl. Acad. Sci. USA*, **105**, 5815–5820.
64 Boden, S.A., Langridge, P., Spangenberg, G., and Able, J.A. (2009) *Plant J.*, **57**, 487–497.
65 Loukoianov, A., Yan, L., Blechl, A. *et al.* (2005) *Plant Physiol.*, **138**, 2364–2373.
66 Pidal, B., Yan, L., Fu, D. *et al.* (2009) *J. Heredity*, **100**, 355–364.
67 Hulbert, S.H., Bai, J., Fellers, J.P. *et al.* (2007) *Genet. Res.*, **97**, 1083–1093.
68 Blum, A., Klueva, N., and Nguyen, H.T. (2001) *Euphytica*, **117**, 117–123.
69 Irmak, S., Naeem, H.A., Lookhart, G.L., and MacRitchie, F. (2008) *J. Cer. Sci.*, **48**, 513–516.
70 Rashidi Asl, A., Mahfoozi, S., and Bihamta, M.R. (2009) *World Acad. Sci. Eng. Tech.*, **49**, 16–18.
71 Pellegrineschi, A., Reynolds, M., Pacheco, M. *et al.* (2004) *Genome*, **47**, 493–500.
72 Selote, D.S., Bharti, S., and Khanna-Chopra, R. (2004) *Biochem. Biophys. Res. Commun.*, **314**, 724–729.
73 Xu, C., Jing, R., Mao, X. *et al.* (2007) *Ann. Bot.*, **99**, 439–450.
74 Pastore, D., Trono, D., Laus, M.N. *et al.* (2007) *J. Exp. Bot.*, **58**, 195–210.
75 Jia, X.Y., Xu, C.Y., Jing, R.L. *et al.* (2008) *J. Exp. Bot.*, **59**, 739–751.
76 Mohammadi, M., Kav, N.N., and Deyholos, M.K. (2008) *Genome*, **51**, 357–367.
77 Kam, J., Gresshoff, P.M., Shorter, R., and Xue, G.P. (2008) *Plant Mol. Biol.*, **67**, 305–322.
78 Ergen, N.Z., Thimmapuram, J., Bohnert, H.J. *et al.* (2009) *Funct. Integr. Genomics*, **9**, 377–396.
79 Mujeeb-Kazi, A., Diaz de, L.J., Ahmed, R., and Malik, K.A. (2002) in *Prospects for Saline Agriculture* (eds R. Ahmad and K.A. Malik), Vol. 37, Kluwer, pp. 69–82.
80 Colmer, T.D., Flowers, T.J., and Munns, R. (2006) *J. Exp. Bot.*, **57**, 1059–1078.
81 Kawaura, K., Mochida, K., Yamazaki, Y., and Ogihara, Y. (2006) *Funct. Integr. Genomics*, **6**, 132–142.
82 Huang, S., Spielmeyer, W., Lagudah, E.S. *et al.* (2006) *Plant Physiol.*, **142**, 1718–1727.
83 Cuin, T.A., Betts, S.A., Chalmandrier, R. *et al.* (2008) *J. Exp. Bot.*, **59**, 2697–2706.
84 Kawaura, K., Mochida, K., and Ogihara, Y. (2008) *Funct. Integr. Genomics*, **8**, 277–286.
85 Mullan, D.J., Mirzaghaderi, G., Walker, E. *et al.* (2009) *Theor. Appl. Genet.*, **119**, 1313–1323.
86 Gulick, P.J., Drouin, S., Yu, Z. *et al.* (2005) *Genome*, **48**, 913–923.
87 Sugie, A., Naydenov, N., Mizuno, N. *et al.* (2006) *Genes Genet. Syst.*, **81**, 349–354.
88 Monroy, A.F., Dryanova, A., Malette, B. *et al.* (2007) *Plant Mol. Biol.*, **64**, 409–423.
89 Kobayashi, F., Ishibashi, M., and Takumi, S. (2008) *Transgenic Res.*, **17**, 755–767.
90 Lobell, D.B. and Field, C.B. (2007) *Environ. Res. Lett.*, **2**.
91 Gallie, D.R., Hanh, L., Caldwell, C. *et al.* (1998) *Biochem. Biophys. Res. Commun.*, **245**, 295–300.
92 Campbell, J.L., Klueva, N.Y., Zheng, H. *et al.* (2001) *Biochim. Biophys. Acta.*, **1517**, 270–277.
93 Gulli, M., Corradi, M. *et al.* (2007) *FEBS Lett.*, **581**, 4841–4849.
94 Qin, D., Wu, H., Peng, H. *et al.* (2008) *BMC Genomics*, **9**, 432.
95 Ristic, Z., Momcilovic, I., Bukovnik, U. *et al.* (2009) *J. Exp. Bot.*, **60**, 4003–4014.
96 Chauhan, H., Khurana, N., Tyagi, A.K. *et al.* (2010) *Plant Mol. Biol.*, in press.
97 Reddy, A.S. (2001) *Plant Sci.*, **160**, 381–404.
98 Wang, Y.J., Yu, J.N., Chen, T. *et al.* (2005) *J. Exp. Bot.*, **56**, 3051–3060.
99 Charron, J.B., Ouellet, F., Pelletier, M. *et al.* (2005) *Plant Physiol.*, **139**, 2017–2028.
100 Egawa, C., Kobayashi, F., Ishibashi, M. *et al.* (2006) *Genes Genet. Syst.*, **81**, 77–91.
101 Faik, A., Abouzouhair, J., and Sarhan, F. (2006) *Mol. Genet. Genomics*, **276**, 478–494.
102 Stephenson, T.J., McIntyre, C.L., Collet, C., and Xue, G.P. (2007) *Plant Mol. Biol.*, **65**, 77–92.
103 Xu, Z.S., Xia, L.Q., Chen, M. *et al.* (2007) *Plant Mol. Biol.*, **65**, 719–732.

104 Brini, F., Hanin, M., Mezghani, I. *et al.* (2007) *J. Exp. Bot.*, **58**, 301–308.

105 Kobayashi, F., Maeta, E., Terashima, A. *et al.* (2008) *J. Exp. Bot.*, **59**, 891–905.

106 Jung, J.H., Hong, M.J., Kim, D.Y. *et al.* (2008) *Genome*, **51**, 856–869.

107 Xu, Z.S., Ni, Z.Y., Liu, L. *et al.* (2008) *Mol. Genet. Genomics*, **280**, 497–508.

108 Shaw, L.M., McIntyre, C.L., Gresshoff, P.M., and Xue, G.P. (2009) *Funct. Integr. Genomics*, **9**, 485–498.

109 Kovacs, Z., Sarkadi, L.S., Szucs, A., and Kocsy, G. (2010) *Amino Acids*, **38**, 623–631.

110 Mao, X., Zhang, H., Tian, S. *et al.* (2009) *J. Exp. Bot.*, **61**, 683–696.

111 Li, A., Wang, X., Leseberg, C.H. *et al.* (2008) *Plant Signal Behav.*, **3**, 654–656.

112 Li, A.L., Zhu, Y.F., Tan, X.M. *et al.* (2008) *Plant Mol. Biol.*, **66**, 429–443.

113 Berna, A. and Bernier, F. (1999) *Plant Mol. Biol.*, **39**, 539–549.

114 Yao, Y., Ni, Z., Zhang, Y. *et al.* (2005) *Plant Mol. Biol.*, **58**, 367–384.

115 Kulshreshtha, R., Kumar, N., Balyan, H.S. *et al.* (2005) *Planta*, **221**, 675–689.

116 Zhao, T., Ni, Z., Dai, Y. *et al.* (2006) *Mol. Gen. Genomics*, **276**, 334–350.

117 Paolacci, A.R., Tanzarella, O.A., Porceddu, E. *et al.* (2007) *Mol. Genet. Genomics*, **278**, 689–708.

118 Kovalchuk, N., Smith, J., Pallotta, M. *et al.* (2009) *Plant Mol. Biol.*, **71**, 81–98.

119 Bellgard, M., Ye, J., Gojobori, T., and Appels, R. (2004) *Funct. Integr. Genomics*, **4**, 1–11.

120 Mullan, D.J., Colmer, T.D., and Francki, M.G. (2007) *Mol. Genet. Genomics*, **277**, 199–212.

121 Boutrot, F., Chantret, N., and Gautier, M.F. (2008) *BMC Genomics*, **9**, 86.

122 Ozdemir, B.S., Hernandez, P., Filiz, E., and Budak, H. (2008) *Int. J. Plant Genomics* **(2008)**, 536104.

123 Vogel, J. and Hill, T. (2008) *Plant Cell Rep.*, **27**, 471–478.

124 Faris, J.D., Zhang, Z., Fellers, J.P. *et al.* (2008) *Funct. Integr. Genomics*, **8**, 149–164.

125 Huo, N., Vogel, J.P., Lazo, G.R. *et al.* (2009) *Plant Mol. Biol.*, **70**, 47–61.

126 Gu, Y.Q., Ma, Y., Huo, N. *et al.* (2009) *BMC Genomics*, **10**, 496.

29
Wheat: Mechanisms and Genetic Means for Improving Heat Tolerance

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Heat stress is one of the major abiotic stresses that reduce crop productivity. Global warming effects are expected to increase the probability and intensity of heat waves, thus exacerbating the existing conditions. In wheat, reduction in yield in hot climates is primarily due to reductions in duration of growth and development. In addition, heat stress results in early leaf senescence and adverse physiological and biochemical changes. There is a strong need to develop crop plants, especially wheat for improved tolerance toward heat stress. This can be achieved by a thorough understanding of (i) various plant responses to high-temperature stress, (ii) by understanding mechanisms of heat stress tolerance, and (iii) by developing possible strategies for enhancing heat tolerance. Adverse affects of heat stress include decline in photosynthesis, increase in photorespiration, reduced water availability, loss of integrity and function of cell membrane, production of reactive oxygen species (ROS), and so on. In order to cope up with stress, plants employ a number of defense mechanisms, which includes overexpression of various enzymatic and nonenzymatic antioxidants to scavenge ROS, maintenance of membrane stability, production of various compatible solutes and metabolites, and induction of various signaling cascades. Understanding all these mechanisms can help us improve heat tolerance in plants using conventional and molecular breeding protocols and transgenic approaches. Heat tolerance in crop plants is reported to have been achieved by genetic engineering of expression of heat shock proteins, increasing the level of osmolytes and various cell detoxification enzymes, and altering membrane fluidity. Considerable variability in thermotolerance has been observed in wheat, especially in wild species including *Aegilops speltoides*. These tolerant species need to be exploited through integration of conventional and molecular breeding approaches. Armed with such wide information and techniques, it will be possible to rationally utilize these for the production of heat-tolerant genotypes with improved productivity.

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29.1 Introduction

As the world population grows exponentially, there is a need to both increase agricultural productivity and expand productive areas into warmer climates of the world. Both of these goals require significant breeding efforts to improve high temperature tolerance of cereal yield and quality. Cereal breeding to date had utilized only a limited number of progenitor and nonprogenitor germplasm resources and emphasized a high-yield potential under favorable environmental conditions narrowing down genetic diversity of stress resistance traits, including heat stress tolerance. Heat stress is a major challenge to wheat productivity in India [1]. Screening of germplasm against environmental stresses, based on physiological parameters and biochemical markers, constitutes a powerful tool to help resolve this problem. Wheat is the most widespread cereal in terms of area planted. Bread and durum wheat (*Triticum aestivum* and *T. durum*) occupy an estimated area of 200 million ha globally. Though India is the second largest producer of wheat in the world, the average productivity is 2770 kg ha^{-1} against 3885 kg ha^{-1} in China and 8043 kg ha^{-1} in the United Kingdom, the Netherlands, and other NW European countries. In India, the lower productivity is due to shorter crop duration and period of grain filling and higher temperatures during crop growth, particularly during grain filling [2].

Continual heat stress affects approximately 7 million ha of wheat in developing countries, while terminal heat stress is a problem in 40% of temperate environments, which cover 36 million ha. Continual heat stress is defined by a mean daily temperature of over 17.5 °C in the coolest month of the season [3], and over 50 countries (importing more than 20 million tons of wheat per year) experience this type of stress throughout the wheat cycle. The food security of India depends to a large extent on wheat crop. Stagnation of wheat yield, declining production in the past few years, after touching the peak of 76.37 million ton in the crop season of 1999–2000, and recent imports have shaken the confidence of the nation, raising apprehensions about the country's ability to produce adequate food to meet the demand of the growing, economically ascendant population. Wheat yield in Punjab declined from 4.7 to 4.2 ton ha^{-1} between 2000 and 2005 [4]. The negative impact of a temperature increase of 0.4 °C observed between 1980 and 2000 on global wheat production has been demonstrated [5]. Average global temperatures are predicted to rise by 2 °C over next 50 years making many wheat growing regions even less suitable, based on predicted temperature ranges.

Wheat (*T. aestivum* L.), like other C_3 species, is not physiologically adapted to perform well under high temperatures. It has been reported that a 1 °C rise in temperature during grain filling shortens this period by 5% and proportionally reduces harvest index and grain yield [6]. Photosynthesis, as measured by carbon exchange rate, was found to be optimum at 25 °C [7]. Yet, temperatures of 35–40 °C are common in many wheat-producing areas of the world [8]. Biomass production can be substantially suppressed by the effects of heat stress on decreasing photosynthesis, impairing respiration, inactivating enzymes, and disrupting

membranes [9]. The adverse effects of heat stress on plant productivity are very pronounced in winter wheat in the southern plains during seedling establishment (late summer) and grain-filling stages (late spring) where high temperatures are common during these critical developmental stages.

29.2 Environmental and Physiological Nature of Heat Stress

Heat stress is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause irreversible damage to plant growth and development. In general, a transient elevation in temperature, usually 10–15 °C above ambient, is considered heat stress. Transient or constant high temperature provoked damage at the morphological, anatomical, physiological, and biochemical levels in plants and may drastically reduce economic yield. High-temperature stress may lead to altered geographical distribution and growing season of agricultural crops by allowing the threshold temperature for the start of the season and crop maturity to reach earlier [10]. At moderately high temperature, injuries or death may occur only after long-term exposure while at very high temperatures, severe cellular injury and even cell death may occur within minutes, which could be attributed to a catastrophic collapse of cellular organization [11]. Direct injuries due to heat stress include protein denaturation and aggregation, and increased fluidity of membrane lipids, whereas indirect or slower heat injuries include inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation, and loss of membrane integrity [12]. Heat stress also affects the organization of microtubule asters in mitotic cells and elongation of phragmoplast microtubules [13]. These injuries eventually lead to starvation, inhibition of growth, reduced ion flux, and production of toxic compounds and reactive oxygen species (ROS) [11].

The extent to which heat stress occurs in specific climatic zones is a complex issue. Plants can be damaged in different ways by either high day or high night temperatures and by either high air or high soil temperatures. In addition, crop species and cultivars differ in their sensitivity to high temperatures. Studies comparing responses of contrasting species to heat indicated that photosystem II of cool season species such as wheat is more sensitive to heat than that of rice and pearl millet, which are adapted to much higher temperatures [14].

In general, heat tolerance can be defined as the ability to maintain integrity of cellular and subcellular structures and metabolic pathways (cellular heat tolerance) that allow continued plant growth and reproduction during and after exposure to heat stress conditions (whole-plant heat tolerance). Immediate exposure to heat stress mediates a cascade of signal transduction that changes the expression of genes and accumulation of transcripts, thereby leading to the synthesis of various stress-related proteins, as a stress tolerance strategy [15]. Expression of various heat shock proteins (HSPs) is known to be a very important adaptive strategy in acquiring heat stress tolerance. These proteins of molecular mass in the range of 10–200 kDa have chaperone-like activities and are involved in the signal transduction during heat

stress [16]. The tolerance conferred by HSPs results in improved physiological phenomena such as photosynthesis, assimilate partitioning, water and nutrient use efficiency, and membrane stability [17]. However, not all the plant species or genotypes within species have similar capabilities to cope with the heat stress. Tremendous variations exist within and between species providing opportunities to improve crop heat stress tolerance through various advanced techniques of molecular breeding and genetic engineering for developing crops with improved heat tolerance and to combat this universal environmental adversary.

29.3 Heat Stress Threshold

A threshold temperature refers to a value of daily mean temperature at which a detectable reduction in growth begins. Upper and lower developmental threshold temperatures have been determined for many plant species through controlled laboratory and field experiments. A lower developmental threshold or a base temperature is one below which plant growth and development stop. Similarly, an upper developmental threshold is the temperature above which growth and development cease. Knowledge of lower threshold temperatures is important both for physiological research and for crop production.

Upper threshold temperatures for some major crop species are presented in Table 29.1. The magnitude of heat stress rapidly increases as temperature increases above a threshold level and complex acclimation effects can occur that depend on temperature and other environmental factors. High-temperature sensitivity is particularly important in tropical and subtropical climates, as heat stress may become a major limiting factor for field crop production. This is because under high temperature conditions, plants tend to divert resources to cope with the heat stress and thus limited photosynthates would be available for reproductive development, which can

Table 29.1 High temperature threshold levels for some crop plants [18].

Crop plant	Threshold temperature (°C)	Growth stage
Wheat	26	Postanthesis
Tomato	30	Emergence
Brassica	29	Flowering
Cool season pulses	25	Flowering
Corn	38	Grain filling
Rice	34	Grain filling
Cotton	45	Reproductive
Pearl millet	35	Seedling
Ground nut	34	Pollen production
Cowpea	41	Flowering

accelerate senescence, diminish seed set and seed weight, and reduce yield [19]. Another effect of heat stress in many plant species is induced sterility when heat is imposed immediately before or during anthesis.

29.4
High-Temperature Impact and Plant Responses to Heat Stress

High temperatures are known to have deleterious effects on photosynthesis, respiration, and reproduction. At molecular level, these effects are brought about by altered gene expression and manifested at the biochemical and metabolic level, membrane stability, and production of heat shock proteins. The latter are postulated to protect organisms from the damaging effects of heat and other forms of stress [20]. Activities of the various enzymes involved in cellular processes are affected by increased temperature. The concept of thermal kinetic window (TKW) of an enzyme is defined as the temperature range in which the Michaelis–Menten constant (K_m) of the enzyme remains within 200% of the optimum. Whole-plant response to temperature stress reflects the thermal dependence of K_m of different enzymes. A 5 °C increase results in selective expression of HSPs, with continued synthesis of normal cellular proteins. With further increase in temperature, synthesis of HSPs predominates, while the others are inhibited. At still higher temperatures, even the synthesis of HSP is inhibited. An earlier study conducted in maize (*Zea mays* L.) kernels demonstrated that heat stress decreases protein accumulation and alters composition of the kernels [21]. Figure 29.1 represents various changes taking place under the influence of heat stress.

29.4.1
Morphological Responses

Heat stress can cause considerable pre- and postharvest damages, including scorching of leaves and twigs, sunburns on leaves, branches, and stems, leaf senescence and abscission, inhibition of shoot and root growth, fruit discoloration and damage, and reduced yield [22]. High temperature-induced responses in plants may differ from one phonological stage to another. For example, long-term effects of heat stress on developing seeds may include delayed germination or loss of vigor, ultimately leading to reduced emergence and seedling establishment. Significant decrease in growth, shoot dry mass, and net assimilation rate has been observed due to prolonged heat stress in several monocotyledons. Heat stress, alone or in combination with drought, is a common constraint during anthesis and grain-filling stages in many cereal crops of temperate regions. For example, heat stress lengthened the duration of grain filling with reduction in kernel growth leading to losses in kernel density and weight by up to 7% in spring wheat [23]. In wheat, both grain weight and grain number appeared to be sensitive to heat stress, as the number of grains per ear at maturity declined with increasing temperature [24]. Reproductive processes are markedly affected by high temperatures in most plants, which ultimately affect fertilization and postfertilization

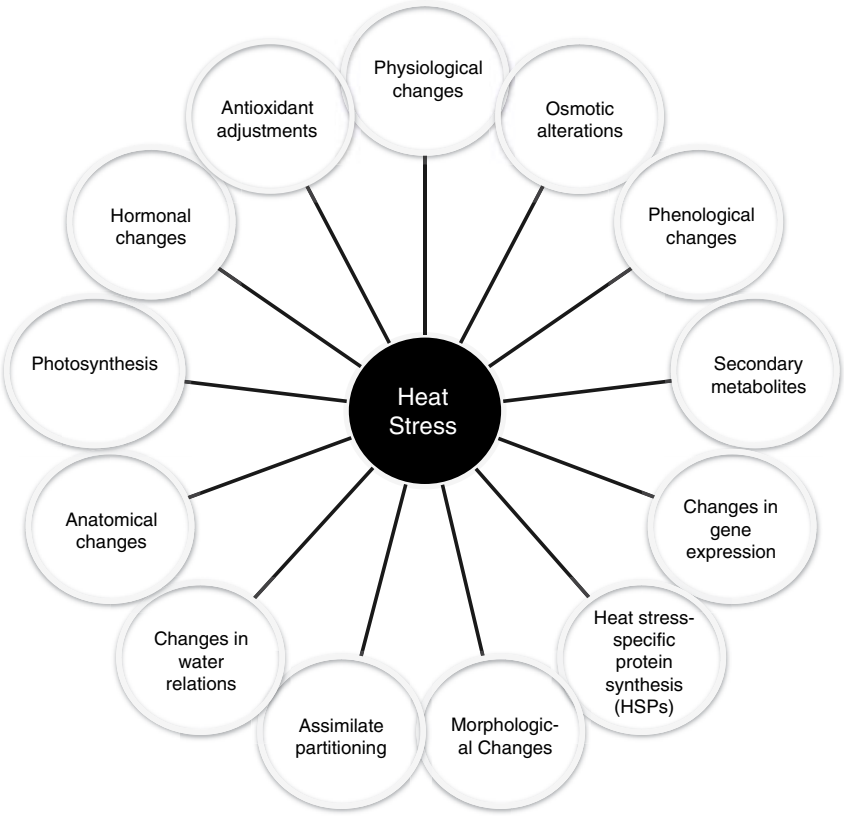

Figure 29.1 Heat stress-induced changes in plants.

processes, leading to a reduced crop yield. A study of postanthesis heat stress on grain yield and quality of Australian wheat reported a decline in individual kernel mass by 23% and alteration in the gliadin:glutenin ratio in response to high temperature treatment. They also reported a considerable genetic variability in wheat in tolerance to short periods of high temperature for both grain yield and quality [25].

29.4.2 Anatomical Responses

It is evident that high temperature considerably affects anatomical structures not only at the tissue and cellular levels but also at the subcellular level. Anatomical changes under high ambient temperatures are generally similar to those under drought stress. At the whole-plant level, there is a general tendency of reduced cell size, closure of stomata, and curtailed water loss, increased stomatal and trichomatous densities, and greater xylem vessels of both root and shoot [26]. At the subcellular level, major modifications occur in chloroplasts, leading to significant changes in

photosynthesis. For example, high temperatures reduced photosynthesis by changing the structural organization of thylakoids [27]. Specific effects of high temperatures on photosynthetic membranes result in the loss of grana stacking or its swelling. Such changes result in the formation of antenna-depleted photosystem II (PSII) and hence reduced photosynthetic and respiratory activities [28].

29.4.3 Phenological Responses

Different phonological stages differ in their sensitivity to high temperature; however, this depends on species and genotype as there are great inter and intraspecific variations. It is, however, not known whether damaging effects of heat episodes occurring at different developmental stages are cumulative. Vulnerability of plant species and cultivars to high temperatures may vary with the stage of development, but all vegetative and reproductive stages are affected by heat stress to some extent. During vegetative stage, for example, high day temperature can damage leaf gas exchange properties. During reproduction, a short period of heat stress can cause significant increases in floral bud and open-flower abortion; however, there are great variations in sensitivity within and among plant species [29, 30]. Impairment of pollen and anther development by elevated temperatures is another important factor contributing to decreased fruit set in many crops at moderate to high temperatures [31]. The staple cereal crops can tolerate only narrow temperature ranges, which if exceeded during the flowering phase can damage fertilization and seed production, resulting in reduced yield [10]. Furthermore, high temperatures during grain filling can modify flour and bread quality and other physicochemical properties of grain crops such as wheat [32], including changes in protein content of the flour [33]. High temperature is one of the major environmental constraints to wheat yield. Heat stress during grain filling is a common occurrence in wheat. High temperatures, typically above 34 °C, affect final grain weight by reducing the duration of grain filling due to suppression of photosynthesis [34] and by directly inhibiting starch biosynthesis in the endosperm [35, 36].

29.4.4 Physiological Responses

Higher temperatures affect all phases of wheat growth, accelerate floral initiation, and reduce the period of spike development, resulting in shorter spike with lower number of spikelets and adversely affecting pollen development. The duration of grain growth in the postanthesis period is considered the most significant determinant of yield in wheat. Higher temperatures further associated with limitation of water cause rapid shrinkage of grain volume. Wheat plants have four to eight leaves on the main shoot when the growing apex changes from the vegetative to the reproductive stage. Temperatures above 30 °C during floret formation cause complete sterility [37, 38]. Previous studies have reported a positive correlation between the length of the vegetative phase and the number of spikelets per spike [39].

Therefore, shortening the duration of the vegetative stage of the apex induces fewer spikelets per spike. The main effect of heat stress after/during floral initiation is observed on kernel number. The numbers of kernels per unit area decreases at a rate of 4% for each degree increase in the mean temperature during the 30 days preceding anthesis [40]. Considerable experimental effort has been devoted to examining carbohydrate availability for developing wheat florets as a major factor in the grain number [41–43]. It has been suggested in previous studies that the time of floret death corresponded with the period when the ear and stem were accumulating dry matter at their most rapid rate and that inadequate assimilate availability may be critical in the loss of florets [44]. It has been well established that wheat yield and quality response to short spells of heat could be improved by heat shock treatment during early grain-filling phase [45].

29.4.5 Water Relations

Plant water status is the most important variable under changing ambient temperatures. Plants tend to maintain stable tissue water status regardless of temperature when moisture is ample; however, high temperatures severely impair this tendency when water is limited [46]. Under field conditions, high-temperature stress is frequently associated with reduced water availability [47]. High temperatures seem to cause water loss in plants more during daytime than during nighttime. During daytime enhanced transpiration induces water deficiency in plants, causing a decrease in water potential and leading to perturbation of many physiological processes.

29.4.6 Osmotic Alterations

A key adaptive mechanism in many plants grown under various abiotic stresses, including extreme temperatures, is accumulation of certain organic compounds of low molecular mass, generally referred to as compatible osmolytes. Under stress, different plant species may accumulate a variety of osmolytes such as sugars and sugar alcohols (polyols), proline tertiary and quaternary ammonium compounds, and tertiary sulfonium compounds [48]. For example, glycine-betaine (GB), an amphoteric quaternary amine and proline, plays an important role as a compatible solute in plants under various stresses, such as salinity or high temperature [49], and has been known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses. It is suggested that proline or GB synthesis may buffer cellular redox potential under heat and other environmental stresses. Similarly, accumulation of soluble sugars under heat stress has been reported in sugarcane, which entails great implications for heat tolerance [50]. Among other osmolytes, γ-4-aminobutyric acid (GABA), a nonprotein amino acid, is widely distributed throughout the biological world to act as a compatible solute. GABA is synthesized from the glutamic acid by a single-step reaction catalyzed by

glutamate decarboxylase (GAD). An acidic pH activates GAD, a key enzyme in the biosynthesis of GABA. Episodes of high temperature increase the cytosolic level of Ca, which leads to calmodulin-mediated activation of GAD. Rapid accumulation of GABA in stressed tissues may provide a critical link in the chain of events stemming from the perception of environmental stresses to timely physiological responses [51].

29.4.7 Photosynthesis

Inhibition of photosynthesis by heat stress is a common occurrence for plants in tropical and subtropical regions and is experienced periodically by plants in the temperate zone [52]. Photochemical reactions in thylakoid lamellae and carbon metabolism in the stroma of chloroplast have been suggested as the primary sites of injury at high temperatures [53]. Increasing leaf temperatures and photosynthetic photon flux density influence heat tolerance adjustments of PSII, indicating their potential to optimize photosynthesis under varying environmental conditions as long as the upper thermal limits do not exceed. A broad survey of the literature reveals that PSII is often considered the most heat-labile component of the photosynthetic apparatus, which may be due to the properties of thylakoid membranes where PSII is located [54]. Heat stress may lead to the dissociation of oxygen evolving complex (OEC), resulting in an imbalance between the electron flow from OEC toward the acceptor side of PSII in the direction of PSI reaction center [55]. Heat stress causes dissociation of manganese (Mn)-stabilizing 33-kDa protein at PSII reaction center complex followed by the release of Mn atoms [56]. In wheat, high temperatures and excessive light damaged different sites of PSII implying different pathways for the recovery of its functional activity [57]. Studies in controlled environments have revealed genetic variability in photosynthetic rate among wheat cultivars when exposed to high temperatures [7, 58]. Such differences in photosynthesis under heat stress have been shown to be associated with a loss of chlorophyll and a change in the a:b chlorophyll ratio due to premature leaf senescence [34, 59].

High temperature influences the photosynthetic capacity of C_3 plants more strongly than in C_4 plants. It alters the energy distribution and changes the activities of carbon metabolism enzymes, particularly the rubisco, thereby altering the rate of RuBP regeneration by the disruption of electron transport and inactivation of the oxygen evolving enzymes of PSII [60]. Heat shock reduces the amount of photosynthetic pigments [61], soluble proteins, rubisco binding proteins (RBP), and large (LS) and small subunits (SS) of rubisco in darkness, but increases them in light, indicating their roles as chaperones and HSPs [62]. Moreover, under heat stress, starch or sucrose synthesis is greatly influenced as activities of sucrose phosphate synthase [63], ADP-glucose pyrophosphorylase, and invertase [64] is significantly reduced.

In any plant species, the ability to sustain leaf gas exchange under heat stress has a direct relationship with heat tolerance. During the vegetative stage, high day temperature can cause damage to compensated leaf photosynthesis, reducing CO_2 assimilation rates. Increased temperatures curtail photosynthesis and increase CO_2

transfer conductance between intercellular spaces and carboxylation sites. Stomatal conductance (g_s) and net photosynthesis (P_n) are inhibited by moderate heat stress in many plant species due to decrease in the activation state of rubisco [65] that catalyzes the first step in two competing pathways, photosynthesis and photorespiration, whose rates are determined by the rates of the carboxylase and oxygenase activities, respectively [66]. The V_{Max} of the carboxylase activity increases with temperature, but the affinity of rubisco for CO_2 and the solubility of CO_2 decrease [67, 68]. A well-known consequence of elevated temperature in plants is the damage caused by heat-induced imbalance in photosynthesis and respiration; in general, the rate of photosynthesis decreases while dark- and photorespiration rates increase considerably under high temperatures. Rate of biochemical reactions also decreases and enzyme inactivation and denaturation take place as the temperature increases leading to severely reduced photosynthesis [69]. However, the magnitude of such alterations in response to heat stress differs with species and genotypes [54]. Furthermore, it has been determined that the photosynthetic CO_2 assimilation rate is less affected by heat stress in developing leaves than in completely developed leaves. Heat stress normally decreases the duration of developmental phases leading to smaller organs, reduced light perception, and reduced carbon assimilation processes including transpiration, photosynthesis, and respiration [70]. Nonetheless, photosynthesis is considered as the physiological process most sensitive to high temperatures, and that rising atmospheric CO_2 content will drive temperature increases in many already stressful environments.

29.4.8 Assimilate Partitioning

Under low to moderate heat stress, a reduction in source and sink activities may occur leading to severe reductions in growth, economic yield, and harvest index. Assimilate partitioning, taking place via apoplastic and symplastic pathways, under high temperatures has significant effects on transport and transfer processes in plants. However, a considerable genotypic variation exists in crop plants for assimilate partitioning, for example, among wheat genotypes [71]. In wheat, temperature effects on translocation result indirectly from temperature effects on source and sink activities. Higher temperatures further associated with limitation of water cause rapid shrinkage of grain volume. Higher temperatures at earlier stages reduce the spike length and the number of spikelets (sink capacity). Heat stress after anthesis reduces the net availability of assimilates, reducing grain number and weight. Increased mobilization efficiency of reserves from leaves, stem, or other plant parts has been suggested as a potential strategy to improving grain filling and yield in wheat under heat stress.

Carbohydrates in wheat grain are derived from postanthesis CO_2 assimilation, of which the flag leaf has a large share. Rubisco constitutes 60% of the soluble protein in the flag leaf. In limiting soil N supply, rubisco becomes the source of N for the developing grains. High turnover of rubisco 10 days after anthesis has been reported. Degradation of rubisco further enhances flag leaf senescence, contributing to

reduction in the supply of assimilates to developing grains. Specific data on the turnover of the HSPs in wheat could not be found, though their degradation and resynthesis in other organisms is known. Therefore, competition for both C and N assimilates between Rubisco, HSPs, and storage proteins in developing grains are expected during continuing heat stress.

29.4.9
Membrane Thermostability

Sustained function of cellular membranes under stress is pivotal for processes such as photosynthesis and respiration. Heat stress accelerates the kinetic energy and movement of molecules across membranes, thereby loosening chemical bonds within molecules of biological membranes. This makes the lipid bilayer of biological membranes more fluid by either denaturation of proteins or an increase in unsaturated fatty acids. The integrity and functions of biological membranes are sensitive to high temperature, as heat stress alters the tertiary and quaternary structures of membrane proteins. Such alterations enhance the permeability of membranes, as evident from increased loss of electrolytes. The increased solute leakage, as an indication of decreased cell membrane thermostability (CMT), has long been used as an indirect measure of heat stress tolerance in diverse plant species including wheat [72]. Three commonly used assays of heat tolerance in plants [73] are related to the plasmalemma ("cell membrane stability" or CMS assay), the photosynthetic membranes (chlorophyll fluorescence assay), and the mitochondrial membranes (cell viability assay based on 2,3,5-triphenyl-tetrazolium chloride (TTC) reduction). Genetic variation in membrane thermostability (MT) has been inferred using conductometric measurements in various field-grown crops including spring wheat [74]. There are reports of a significant increase in yield of spring wheat in hot locations by selecting membrane thermostable lines, as determined by measurements on flag leaves at anthesis [75].

29.4.10
Hormonal Modulations, Secondary Metabolites, and Antioxidant Adjustments

Hormones play an important role in plants for monitoring and adapting under adverse environmental conditions. Hormonal homeostasis, stability, content, biosynthesis, and compartmentalization are altered under heat stress [76]. Abscisic acid (ABA) is implicated in plant osmotic stress responses and mediates one of the intracellular dehydration signaling pathways [77]. In the field, where heat and drought stresses frequently occur simultaneously, ABA induction can be an important component of heat tolerance. ABA mediates acclimation/adaptation of plants to desiccation by modulating the up- or downregulation of numerous genes [78]. Induction of several HSPs by ABA could be one mechanism by which it confers tolerance to heat stress [79] and can cooperate with other HSPs to reactivate heat-denatured normal proteins [80]. However, the effects of gibberellins and cytokinins on high temperature tolerance are opposite to that of ABA.

A gaseous hormone, ethylene, regulates almost all growth and developmental processes in plants, ranging from seed germination to flowering and fruiting, as well as tolerance to environmental stresses. Ethylene has nearly full biological activity at 1 μl l^{-1}, corresponding to 6.5×10^{-9} M at 25 °C. Heat stress changes ethylene production differently in different plant species [81]. For example, while ethylene production in wheat leaves was inhibited slightly at 35 °C and severely at 40 °C, in soybean ethylene production in hypocotyls increased by increasing temperature up to 40 °C and it showed inhibition at 45 °C. Wheat leaves transferred to 18 °C followed by a short exposure to 40 °C showed an increase in ethylene production after 1 h lag period, possibly due to conversion of accumulated ACC to ethylene during that period [82]. Among other hormones, salicylic acid (SA) has been suggested to be involved in heat stress responses elicited by plants. SA is an important component of signaling pathways in response to systemic acquired resistance (SAR) and the hypersensitive response (HR) [83]. SA stabilizes the trimers of heat shock transcription factors (HDFs) and aids them bind heat shock elements (HSEs) to the promoter of heat shock-related genes. Long-term heat tolerance can be induced by SA, in which both Ca^{2+} homeostasis and antioxidant systems are thought to be involved [84]. Sulphosalicylic acid (SSA), a derivative of SA, treatment can effectively remove H_2O_2 and increase heat tolerance.

Heat stress causes accumulation of secondary metabolites of multifarious nature in plants. Most of the secondary metabolites are synthesized from the intermediates of primary carbon metabolism via phenylpropanoid, shikimate, mevalonate, or methyl erythritol phosphate (MEP) pathways. High-temperature stress induces production of phenolic compounds such as flavonoids and phenylpropanoids. Phenylalanine ammonia-lyase (PAL) is considered to be the principal enzyme of the phenylpropanoid pathway. Increased activity of PAL in response to thermal stress is considered as the main acclamatory response of cells to heat stress. Thermal stress induces the biosynthesis of phenolics and suppresses their oxidation, which is considered to trigger the acclimation to heat stress.

Carotenoids are widely known to protect cellular structures in various plant species irrespective of the stress type [18]. For example, the xanthophyll cycle (the reversible interconversion of two particular carotenoids, violaxanthin and zeaxanthin) has evolved to play this essential role in photoprotection. Since zeaxanthin is hydrophobic, it is found mostly at the periphery of the light harvesting complexes, where it functions to prevent peroxidative damage to the membrane lipids triggered by ROS [85]. Recent studies have revealed that carotenoids of the xanthophylls family and some other terpenoids, such as isoprene or α-tocopherol, stabilize and photoprotect the lipid phase of the thylakoid membranes. When plants are exposed to potentially harmful environmental conditions, such as strong light and/or elevated temperatures, the xanthophylls including violaxanthin, antheraxanthin, and zeaxanthin partition between the light-harvesting complexes and the lipid phase of the thylakoid membranes. The resulting interaction of the xanthophyll molecules and the membrane lipids brings about a decreased fluidity (thermostability) of membrane and a lowered susceptibility to lipid peroxidation under high temperatures.

Phenolics, including flavonoids, anthocyanins, lignins, and so on are the most important class of secondary metabolites in plants and play a variety of roles including in tolerance to abiotic stresses [18]. Studies suggest that accumulation of soluble phenolics under heat stress was accompanied by increased phenyl ammonia lyase (PAL) and decreased peroxidase and polyphenol lyase activities. Another plant secondary product, which is a low molecular weight and volatile in nature, called isoprenoid confers heat stress tolerance to photosynthesis apparatus in different plants. It is proposed that endogenous production of isoprene protects the biological membranes from damaging effects by directly reacting with oxygen singlets ($^1O_2$) by means of isoprene-conjugate double bond.

High temperature-induced oxidative stress in various higher and lower plants has been reported by many workers [86, 87]. Generation and reactions of activated oxygen species (AOS) including singlet oxygen ($^1O_2$), superoxide radical ($O^{2\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\cdot^-$) are symptoms of cellular injury caused by high temperature [88]. Active oxygen species cause lipid peroxidation and consequently membrane injury, protein degradation, enzyme inactivation, pigment bleaching, and disruption of DNA strands and thus limiting growth and yield [89]. Superoxide radical is regularly synthesized in the chloroplast and mitochondrion and some quantities are also produced in microbodies. The scavenging of O^{2-} by superoxide dismutase (SOD) results in the production of H_2O_2, which is removed by APX or CAT. However, both O^{2-} and H_2O_2 are not as toxic as the (OH^-), which is formed by the combination of O^{2-} and H_2O_2 in the presence of trace amounts of Fe^{2+} and Fe^{3+} by the Haber–Weiss reaction. Plants protect cellular and subcellular systems from cytotoxic effects of these active oxygen radicals using antioxidant enzymes such as glutathione reductase, superoxide dismutase, ascorbate peroxidase, and catalase and metabolites such as glutathione, ascorbic acid, α-tocopherol, and carotenoids [90]. Tolerance to heat stress in crop plants has been reported to be associated with an increase in antioxidant enzyme activities [91]. It has also been reported that heat stress in wheat during greening led to inactivation of PS2 vis-a-vis high turnover of both PS1 and superoxide dismutase, which allowed formation of superoxide and its dismutation product, H_2O_2, and reversible modulation of enzymatic H_2O_2-scavenging suggesting a central role of H_2O_2 metabolism in seedling response to thermal stress [92].

29.5
Heat-Induced Protein Synthesis

To cope with heat stress, plants activate a large set of genes leading to the accumulation of specific stress-associated proteins [93]. Expression of stress proteins is an important adaptation toward heat-stress tolerance by plants. Of these, expression of low and high molecular weight HSPs, widely reported in a number of plant species, is the most important one. These proteins show organelle- and tissue-specific expression with deduced function such as chaperones, folding and unfolding of cellular proteins, and protection of functional sites from the adverse effects of high

temperature. Most of the stress proteins are soluble in water and therefore contribute to stress tolerance presumably via hydration of cellular structures.

29.5.1 Heat Shock Proteins

Plants respond to high-temperature stress by synthesizing an assortment of proteins, termed heat shock proteins. Certain HSPs are expressed under certain stages of development such as embryogenesis, germination, pollen development, and fruit maturation [94]. HSP-triggered heat tolerance is attributed to the observations that (a) their induction coincides with the organism under stress, (b) their biosynthesis is extremely fast and intensive, and (c) they are induced in a wide variety of cells and organisms. Among five conserved families of Hsps (Hsp100, Hsp90, Hsp70, Hsp60 and sHsp), the small heat shock proteins (sHsps) are found to be most prevalent in plants, the expression of which can increase up to 200-fold under heat stress. sHsps vary in size from 12–40 kDa [95]. All sHSPs in plants are encoded by six nuclear gene families, with each gene family corresponding to proteins found in distinct cellular compartments such as cytosol, chloroplast, endoplasmic reticulum (ER), mitochondria, and membranes. The major chaperone activity of sHsps is to bind and hold denatured substrates in a folding-competent state for subsequent refolding by a chaperone network. However, some members of the plant sHsps can also stabilize or reactivate inactivated enzymes.

The mechanism by which HSPs contribute to heat tolerance is still enigmatic though several roles have been ascribed to them. Many studies assert that HSPs are molecular chaperones ensuring the native configuration and functionality of cell proteins under heat stress. There is considerable evidence that acquisition of heat tolerance is directly related to the synthesis and accumulation of HSPs [96]. For instance, HSPs provide for new or distorted proteins to fold into shapes essential for their normal functions. They also help shuttling proteins from one compartment to another and transporting old proteins to "garbage disposals" inside the cell. The HSPs were recognized to protect normal proteins from the direct effects of denaturation during heat stress treatment. Earlier studies also reported a similar correlation between the thermostabilization of normal proteins and the amount of HSPs synthesized [97]. Among others, HSP70 has been extensively studied and is proposed to have a variety of functions such as protein translation and translocation, proteolysis, protein folding or chaperoning, suppressing aggregation, and reactivating denatured proteins. In many plant species, heat tolerance of cells and tissues after a heat stress is pretty much dependent upon induction of HSP70, though HSP101 has also been shown to be essential [11]. HSP70 participates in ATP-dependent protein unfolding or assembly/disassembly reactions and it prevents protein denaturation during heat stress. Evidence for the general protective roles of HSPs comes from the fact that mutants unable to synthesize them or the cells in which HSP70 synthesis is blocked or inactivated are more susceptible to heat injury [98]. LMW-HSPs play structural roles in maintaining cell membrane integrity. Localization

of LMW-HSPs in chloroplast membranes further suggested that these proteins protect the PSII from adverse effects of heat stress and play a role in photosynthetic electron transport.

There is accumulating evidence that HSPs play an important role in heat tolerance of wheat. A wheat ditelosomic line lacking the long arm of chromosome 1B, DT1BS, was able to acquire heat tolerance at lower induction temperatures and was therefore better protected from heat stress damage [99]. Coincidentally, expression of HSPs in this line was induced at temperatures 4 °C lower than those required for HSP induction in the original wheat cultivar Chinese Spring. Hence, the long arm of chromosome 1B is likely to carry genes repressing both the heat shock response (HSR) at lower temperature and heat tolerance. In contrast, a ditelosomic line missing the long arm of chromosome 7D was heat sensitive and exhibited reduced expression of some HSPs; it was concluded that it carries genes necessary for induction of several HSPs and the capacity to acquire heat tolerance [100].

29.5.2
Other Heat Stress Proteins

Besides HSPs, there are a number of other plant proteins, including ubiquitin, cytosolic Cu/Zn-SOD, and Mn-POD whose expressions are stimulated upon heat stress. For example, in *Prosopis chilensis* and soybean under heat stress, ubiquitin and conjugated ubiquitin synthesis during the first 30 min of exposure emerged as an important mechanism of heat tolerance [101]. A number of osmotin-like proteins induced by heat and nitrogen stresses, collectively called Pir proteins, have also been found to be overexpressed in many plant cells under heat stress conferring them resistance.

Late-embryogenesis abundant (LEA) proteins can prevent aggregation and protect the citrate synthase (an important enzyme of TCA cycle) from desiccating conditions such as heat and drought stress (Figure 29.2). Using proteomics tool, enhanced expressions of 25 LEA proteins in hexaploid wheat during grain filling has been reported [102]. LEA-type proteins fall into a number of families, with diverse structures and functions [103]. Predictions of secondary structures suggest that most LEA proteins exist as random coiled α-helices. It was therefore proposed that most LEA and dehydrin proteins exist as largely unfolded structures in their native state, although a few members exist as dimers or tetramers. Function of these proteins is apparently related to protein degradation pathway, minimizing the adverse effects of dehydration and oxidative stress during heat stress. Hydrophilicity is a common characteristic of LEA-type and other osmotic stress-responsive proteins. Heat stability is another notable feature of LEA proteins, that is, they do not coagulate upon boiling. Another common characteristic of LEA-type proteins is that, in most cases, their related gene expression is transcriptionally regulated and responsive to ABA. It has been suggested that LEA-type proteins act as water binding molecules, both in ion sequestration and in macromolecule and membrane stabilization (i.e., chaperone-like activity).

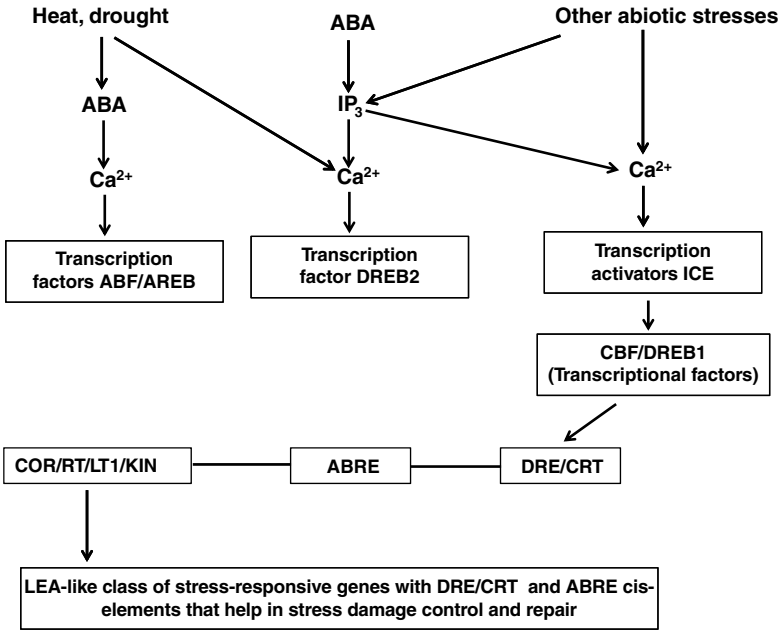

Figure 29.2 LEA proteins activation in response to abiotic stresses.

29.6 Mechanisms of Heat Tolerance

Heat tolerance includes the ability to withstand growth and developmental phase acceleration and to maintain economic yields. A major effect of heat on plants is the acceleration of plant growth and development phases by high temperatures [73]. If the acceleration is severe, economic yield can be affected. Plants adapt to heat stress through long-term evolutionary manifestation of developmental and morphological changes and short-term adaptation mechanisms such as spirational cooling and changes in composition of membrane lipids. Under heat stress, many crop plants employ early maturation as one of the surviving mechanisms that results in reduced yield. Initial effects of heat stress, which include plasma membrane disruption, osmotic changes, and ionic effects, may initiate a downstream signaling and transcriptional cascade that activate stress-responsive mechanism for the reestablishment of the homeostasis and repair of the damaged proteins and membranes. Delayed or poor responses at any step in the signaling and gene activation processes may result in irreversible damage to cellular homeostasis and destruction of functional and structural proteins and membranes, leading to cell death [104, 105]. Various types of stresses may be encountered by a plant at various developmental stages, and the mechanism of the stress response may be tissue specific. [106]; however, the effect of heat stress is not limited to just one level or organelle [107], but the initial effects are on plasma membrane whose fluidity increases under stress

triggering Ca^{2+} influx and cytoskeletal reorganization resulting in the activation of some mitogen-activated protein kinases (MAPK) and calcium-dependent kinases (CDPK). This leads to a series of changes and heat tolerance mechanisms such as ion transporters, osmoprotectants, free radical scavengers, late-embryogenesis abundant proteins, and factors involved in signaling cascades and transcriptional control are essentially significant to counteract the stress effects [108]. Generation of ROS is accompanied by membrane damage; however, these play a crucial role in signaling cascade. In wheat genotypes, increased activity of SOD and CAT and high ascorbic acid content has been well correlated with acquiring heat tolerance by overcoming the oxidative damage [48]. Induction of HSPs along with other proteins (such as LEA and dehydrins) is another mechanism of acquiring heat tolerance. In addition to their chaperone-like activity, they act as signaling molecules and interact with other stress response mechanisms such as osmolytes and antioxidants [109, 110]. The significance of HSPs in heat tolerance was first mooted on the basis of correlative evidence (for a review, see Refs [95, 111]), but the involvement of several HSPs in the acquired heat tolerance of *Arabidopsis* is now well demonstrated [106, 112, 113]. HSPs are also involved in stress signal transduction and gene activation, in protecting photosynthetic electron transport [114], and in maintaining cellular redox state [115, 116].

Heat stress is known to swiftly alter patterns of gene expression [117], stimulating the expression of HSP complements and suppressing the expression of many other genes [118], resulting in a heat shock response, which is defined as a transient reprogramming of gene expression and is a conserved biological reaction of cells and organisms to elevated temperatures [11]. The mRNAs encoding non heat stress-induced proteins are destabilized during heat stress. Heat stress may also inhibit splicing of some mRNAs. Heat stress transcription factors (HSFs) are the terminal components of signal transduction pathways mediating the activation of genes (including their own) responsive to heat stress. Heat tolerance is not controlled by a single "thermotolerant" gene in cereals. Different components of tolerance determined by different sets of genes are critical for heat tolerance at different stages of the life cycle and in various tissues. Quantitative trait loci analysis, correlation, and cosegregation approaches, and the use of genetic stocks are most applicable to the dissection of the genetic basis of heat tolerance in cereals.

Genetic diversity for heat tolerance in cultivated wheat is well established [119–122]. Genetic diversity for heat tolerance has been shown to exist in wild *Triticum* and *Aegilops* species in a study in which accessions from Afghanistan, Iran, Iraq, Israel, Jordan, Syria, Lebanon, Turkey, and the former USSR were tested [123]. It was found that all heat-tolerant accessions came from only three regions: Eastern Israel, Western Jordan, and South-western Syria. Hence, it was suggested that the bread and durum wheat landraces from these regions might provide genotypes with a high degree of heat tolerance that could be incorporated into modern wheat backgrounds.

Heat tolerance can be acquired by prior exposure to a conditioning pretreatment, which can be a short but sublethal high temperature or it may also be induced by a gradual increase in temperature to lethal highs, as would be experienced under natural conditions [95]. This protects cells and organisms from a subsequent lethal heat stress. It has been well established that quality and yield responses of wheat

genotypes to short spells of heat could be considerably improved by a heat shock treatment during the early grain-filling phase [45].

29.6.1 Signal Transduction under Heat Stress

In order to achieve heat stress tolerance, multiple stress signaling cascade pathways play key role. Some of these pathways are common to various abiotic stresses while some are stress specific [124]. Various signaling molecules such as ROS, Ca^{2+}, and hormones reprogram the genetic information via signaling cascades [125]. Various heat stress sensors are thought to be located in thylakoid membrane and are capable of detecting physical phase transition and eventually leading to conformational changes in membrane through cycles of phosphorylation and dephosphorylation due to temperature changes [126]. The presence of highly unsaturated lipids and temperature-sensitive photosystem make thylakoid membrane a crucial heat stress sensor [107].

Heat stress signaling involves various chemical molecules. As a response to heat stress, cytosolic Ca^{2+} rises sharply and activates the MAPK, which in turn activates various transcriptional factors via phosphorylation cascade, which then regulates the expression of genes involved in stress adaptation. In other pathway, Ca^{2+} binding activates the calmodulin that in turn activates CDPKs, which are found to be involved in the regulation of the expression of various HSP genes. It is well established that increasing cytosolic Ca^{2+} content under heat stress may alleviate heat injury, increase the activity of antioxidants [127], maintain turgor in the guard cells [128], and enable plant cells to better survive. However, excessive Ca^{2+} released into the cytosol and sustained high cytosolic Ca^{2+} concentration might be cytotoxic [129]. Other signaling molecules include SA, ACC, ABA, and H_2O_2, which are involved in reducing oxidative damage to the cell and hence help in providing heat tolerance to the plant. Although numerous molecules including ROS, hormones, nitrous oxide, and ethylene have been identified for the perception of heat stress cues, the role of Ca^{2+} is most important.

29.6.2 Genetic Approaches to Combat Heat Stress

When a plant is subjected to heat stress, a number of genes are turned on, resulting in increased levels of several metabolites and proteins, some of which may be responsible for conferring a certain degree of protection against these stresses. A key to progress toward breeding better crops under stress has been to understand the changes in molecular machinery that occur in response to stress. Various stress responses accommodate short-term reaction or tolerance to specific stresses. However, genome plasticity in plants, including genetic (e.g., directed mutation) and epigenetic (e.g., methylation, chromatin remodeling, and histone acetylation) changes, allows long-term adaptation to environmental changes/conditions [130], which may be necessary for long-term survival of plant genotypes/species in

particular environmental niches. The use of various biotechnological approaches provides a way to reduce the losses caused by high temperature, but it requires sound biological knowledge of particular organism and the mechanism of tolerance. With the advances in genome sequencing techniques, we have the genomic information of various cereal crops (e.g., maize, rice, and sorghum) that can be exploited to improve heat tolerance in crop species. Cultural practices such as planting time, planting methods, and soil and irrigation management have long been in use to minimize the stress effects. However, in practice, to be successful in improving agricultural productivity in stress environments, both genetic improvement and adjustment in cultural practices must be employed simultaneously the advent of recombinant DNA (rDNA) technology methods has opened avenues for tackling issues relating to complex genetic traits. In particular, the application of quantitative trait locus (QTL) mapping has contributed to a better understanding of the genetic relationship among tolerances to different stresses.

29.6.3
Conventional Breeding

Most plant breeding programs have been focused on the development of cultivars with high-yield potential in favorable (i.e., nonstress) environments. Such efforts have been very successful in improving the efficiency of crop production per unit area and have resulted in significant increases in total agricultural production [131]. The progress in breeding for stress tolerance depends upon an understanding of the physiological mechanisms and genetic bases of stress tolerance at the whole plant, cellular, and molecular levels, for which considerable information is available regarding the physiological and metabolic aspects of plant heat stress tolerance, as discussed earlier. Selection of tolerant genotypes under field conditions is not a viable approach due to the presence of various environmental factors that affects the results in the repeated trials and stage-specific responses making it difficult to correlate the results of one developmental stage with other. One of the traditional breeding methods for the selection of heat-tolerant progenies is to grow the breeding material in the heat-prone environment and to screen the lines with greater yield capacity, but the presence of other abiotic and biotic stresses make the selection process quite difficult. To overcome this problem, one effective strategy is the use of glasshouses for selecting heat-tolerant material, where the required temperature conditions can be maintained effectively throughout the experiment. But still the efficient and reliable selection method for the screening of heat-tolerant genotypes is a major challenge in traditional breeding. Several other selection methods have also been adopted such as a heat tolerance index (HTI), which is a ratio of the increase in coleoptile length after a finite exposure to heat stress (e.g., at 50 °C) to the increase in coleoptiles length in the no-stress treatment, first described in sorghum. This is a very cost-effective and easy-to-assay technique of screening for heat tolerance, but its correlation with performance under field conditions and its effectiveness in different crop species are yet unknown [132]. Pollen viability has been suggested as an additional indirect selection criterion for heat tolerance.

Wheat cultivars capable of maintaining high 1000-kernel weight under heat stress appear to possess higher tolerance to hot environments [122]. Physiological traits that are associated with wheat yield in heat-prone environments are canopy temperature depression, membrane thermostability, leaf chlorophyll content during grain filling, leaf conductance, and photosynthesis [122]. Breeding programs may measure such traits to assist in the selection of heat-tolerant parents, segregating generations, or advanced lines. In a previous study, canopy temperature depression was used to select for yield under a hot, dry, and irrigated wheat environment in Mexico [133], whereas another study reported that leaf chlorophyll content was correlated with 1000-kernel weight while screening Mexican wheat landraces [134]. Such sources of alleles coupled with some of the above traits can provide means for genetically enhanced wheat by design in heat-prone environments. Multidisciplinary research involving genetic resource enhancement and crop physiology at CIMMYT has led to a physiological trait-based approach to breeding for abiotic stress that has merit over breeding for yield per se by increasing the probability of successful crosses resulting from additive gene action. Advances have already been made in the drought breeding program [135, 136], and this strategy will be used to breed wheat for the high-temperature stress environments.

Traditional breeding strategies for heat stress tolerance have attempted to utilize genetic variation arising from varietal germplasm, interspecific or intergeneric hybridization, induced mutations, and somaclonal variation in cell and tissue cultures have met with only limited success due to several reasons, for example, lack of suitable source of genes in sexually compatible gene pools, complexity of the heat stress tolerance trait, lack of understanding of the genetic mechanisms of the high-temperature tolerance response, low genetic variance of yield components under stress conditions, and lack of efficient selection techniques. However, classical breeding prove more effective when blend with various biotechnological approaches to seek the natural sources of heat stress tolerance. Despite all the complexity of heat tolerance and difficulties encountered during transfer of tolerance, some heat-tolerant inbred lines and hybrid cultivars with commercial acceptability have been developed and released, at least in a few crop species such as tomato [137, 138].

29.6.4
Molecular Approaches

It has been well established by various traditional and transgenic approaches that plant heat stress tolerance is a multigenic trait, that is, heat tolerance components are controlled by different set of genes at different developmental stages of plant or in different tissues [105]. Thus, the use of genetic stocks with different degrees of heat tolerance, correlation and cosegregation analyses, molecular biology techniques, and molecular markers to identify tolerance QTL are promising approaches to dissect the genetic basis of heat tolerance [76]. Quantitative trait loci analysis is a powerful tool for qualitative and quantitative genetic analysis of complex traits [139, 140]. Simple sequence repeats, also called microsatellites, were interspersed ubiquitously in the DNA of hexaploid wheat [141]. The PCR-based, sequence-tagged site markers

detected a higher level of genetic variation than did RFLP and RAPD [142–145] and were useful for genetic analysis of species such as hexaploid wheat that exhibit a narrow genetic base due to their recent origin [141, 146]. The chromosome-specific feature of the SSR markers was also valuable for localizing linked alleles and detecting the QTL of interest. Wheat microsatellites were used recently for mapping genes in hexaploid wheat [147], characterizing the identity of genetic stocks [148], studying the genetic diversity of hexaploid wheat and related species [143, 149], and identifying QTL that control grain protein content [150] and preharvest sprouting [151]. High temperature-induced gene expression system is one of the best-studied model systems for analyzing induced gene expression. In recent years, detailed understanding has been gained on various components of the HSR in living organisms including features such as heat shock genes/proteins, heat shock promoters, and heat shock elements (HSEs), heat shock factors (HSFs), possible receptors of the heat shock response, signaling components, and chromatin remodeling aspects [152, 153]. It has been determined that induction of many heat-inducible genes is attributed to the conserved HSEs, which are located in the TATA box proximal 5′ flanking regions of heat shock genes [11]. A number of other sequence motifs have also been identified in plants that show quantitative effect on the expression of heat shock genes such as CCAAT box and AT-rich sequences [154].

Marker-assisted selection (MAS) and genetic engineering are two most common molecular approaches for improving stress tolerance in plants. With the advent of molecular markers, MAS has become an essential component of new discipline termed as "molecular breeding," with the help of which allelic variation among the genes underlying traits can be precisely and efficiently detected [155]. Numerous markers such as RAPDs, AFLPs, RFLPs, and SSRs have been reported for various abiotic stresses for achieving mapping of the QTL involved in stress tolerance [156]. Comparatively, however, limited research has been conducted to identify genetic markers associated with heat tolerance in different plant species. In *Arabidopsis*, for example, four genomic loci (QTL) determining its capacity to acquire heat tolerance were identified using a panel of heat-sensitive mutants [113].

29.6.5
Transgenic Approach

In contrast with traditional breeding and marker-assisted selection programs, the direct introduction of a small number of genes by genetic engineering seems to be a more attractive and rapid approach for improving stress tolerance. Present engineering strategies rely on the transfer of one or several genes that encode either biochemical pathways or endpoints of signaling pathways that are controlled by a constitutively active promoter. These gene products protect, either directly or indirectly, against environmental stresses. The need for raising high-temperature-tolerant crops using recombinant DNA methods was felt since the early days of recombinant DNA science, but not much could be achieved as the underlying physiological processes, biochemical enzymes, and molecular mechanisms that

impart high temperature tolerance were not precisely understood. But recently, many studies have shown that plants for high-temperature tolerance can be genetically engineered by altering Hsps either directly or through regulatory circuits that govern Hsp levels, levels of osmolytes, components of the cell detoxification mechanisms, and components that regulate membrane fluidity [157, 158]. Transgenic technology has emerged as a useful tool for improving genetics of crops for better survival, growth, and yield [159]. During the past one decade, major success has been achieved in producing transgenic plants with increased tolerance to different abiotic stresses [159]. However, not much progress has been made in producing transgenic wheat plants tolerant to heat stress. Table 29.2 provides comprehensive details on some plant transgenics raised for high-temperature tolerance. During the past nearly 30 years of research, Hsps have been extensively analyzed for their physiological, biochemical, cellular, and molecular properties [160, 161].

Hsp are believed to be important for the protection of cells against heat injury both in basal heat tolerance (i.e., heat tolerance achieved without prior HS) and in acquired heat tolerance responses. Over the years, a large number of *hsp* genes have been isolated, sequenced, and cloned [161, 162]. Experimental data obtained from transgenics, reverse genetics, and mutation approaches in noncereal species confirm causal involvement of HSPs in heat tolerance in plants [106]. Several groups have altered levels of sHsps in bacterial cells and plants and have shown that overexpression of sHsps has a role in conferring heat tolerance. For example, it has been shown that overexpression of *Oshsp16.9* in *Escherichia coli* confers heat tolerance to bacterial cells [163]. The involvement of Hsps in regulating heat tolerance in plants has been indicated by downregulating their levels through antisense and RNAi approach. Mutants of *Z. mays* and *A. thaliana* plants underexpressing their respective Hsp100 proteins are observed to lack both basal and induced heat tolerance [113, 164, 165]. Heat shock elements interact with positively acting regulatory HSF proteins to bring about increased transcription of *hsp* genes [166]. In recent years, *hsf* gene induction system has emerged as a powerful target for manipulating levels of Hsps in transgenic experiments.

Various osmolytes such as amino acids (e.g., proline), polyamines (e.g., putrescine), quaternary ammonium compounds (e.g., glycine-betaine), sugars (e.g., mannitol, fructans, sorbitol, and trehalose), and sugar alcohols (e.g., polyols) help plants to acclimatize largely against the osmotic stresses [167]. Many heat stress-tolerant transgenic plants have also been raised by altering osmolytes. Development of plants capable of higher production of glycine-betaine through transformation with the *BADH* gene has been suggested as a potentially effective method to enhance heat tolerance in plants [168]. Overaccumulation of glycine-betaine has been shown to improve heat tolerance in transgenic wheat line T6 due to increased osmotic adjustment and improved antioxidant defense system including antioxidative enzymes and antioxidants [169].

Altering membrane fluidity provides another means for producing heat-tolerant plants. For example, transgenic tobacco plants with altered chloroplast membranes by silencing the gene encoding chloroplast omega-3 fatty acid desaturase have been produced that produce less trienoic fatty acids and more dienoic fatty acids in their

Table 29.2 Transgenic attempts to enhance plant temperature stress tolerance.

Gene	Host	Function	Target	Phenotype	Reference
Zmeftu1	*T. aestivum*	Chloroplast protein synthesis elongation factor, Ef-Tu	Constitutive overexpression	Heat shock tolerant	[174]
Fad7	Tobacco	Fatty acid desaturation	Cosupression	Heat shock tolerant	[170]
Hsf3	*Arabidopsis*	Transcription factor	Constitutive overexpression	Heat shock tolerant	[110]
Hsp101	Maize	HSP	Knockout	Abolished acquired heat tolerance	[165]
Atshf1	*Arabidopsis*	Transcription factor	Heat shock factor	High-temperature tolerant	[175]
HvAPX1	Barley	Photoproduced H_2O_2 detoxification	Ascorbate peroxidase	Heat shock tolerant	[172]
Fad8	Oilseed rape	Oxidoreductase acting on paired donors with incorporation or reduction of molecular oxygen	Desaturase enzyme	High-temperature tolerant	[28]
Hsp17.7	Carrot	HSP	Constitutive overexpression or antisense	Mild tolerant to high temperature	[176]
Fad7	Tobacco	Fatty acid desaturation	Constitutive overexpression in chloroplast	Increased levels of 16:3 and 18:3 fatty acids, HS tolerant	[177]

chloroplasts than do the wild type. These plants exhibited greater photosynthesis and grew better than wild type plants under high temperatures [170]. Molecular manipulations focused on the component of cell detoxification mechanisms have been employed in specific experiments to alter heat tolerance response in transgenic plants. Reactive oxygen species are induced by most types of stresses [48, 171] and their production has been envisaged in stress cross-tolerance. Overexpression of barley *hvapx1* gene (encoding for peroxisomal ascorbate peroxidase) in *Arabidopsis* brought about increased heat tolerance in transgenic plants compared to wild-type plants [172]. Overexpression of Cu/Zn superoxide dismutase is also noted to protect plants from high-temperature stress [173]. Details of the transgenic events produced for improving heat tolerance in plants are presented in Table 29.2.

29.6.6
Functional Genomics of Heat Stress

Heat stress is not a single-step event. Rather, it triggers in plants a cascade of physiological, metabolic, and molecular events. The long history of breeding, genetics, and physiology of crops provides a unique resource for genomics studies in these species. Genomics, proteomics, and metabolomics, coupled with a strong bioinformatics capability, now enable a "broad" approach to be taken in the study of plant responses to abiotic stresses. Recent advances in genome-wide analyses have revealed complex regulatory networks that control global gene expression, protein modification, and metabolite composition. Genetic regulation and epigenetic regulation, including changes in nucleosome distribution, histone modification, DNA methylation, and npcRNAs (nonprotein-coding RNAs) play important roles in heat stress gene networks. Transcriptomics, metabolomics, bioinformatics, and high-throughput DNA sequencing have enabled active analyses of regulatory networks that control abiotic stress responses. Such analyses have markedly increased our understanding of global plant systems in responses and adaptation to heat stress conditions.

Functional genomics technologies provide tools for the detection and definition of cellular networks through which stress perception, signal transduction, and defensive responses are mediated. This understanding can be used for the manipulation of the responses or their transfer to important cereal crops species through either conventional, marker-assisted, or transgenic approaches (Figure 29.3). Various genes and gene products, which are involved in signal transduction and eventually gene expression involved in heat stress tolerance mechanism, have been elucidated by both forward and reverse genetics approaches [178].

Functional genomics and proteomics have emerged as a powerful tool in understanding the various mechanisms behind the heat tolerance and eventually in producing heat-tolerant genotypes. Various techniques such as TILLING, antisense RNA/RNA interference, posttranscriptional gene silencing, virus-induced gene silencing, gene knockout, and T-DNA insertional mutagenesis have revolutionized our understanding of the function of various genes involved in heat stress response [179] (Figure 29.4). Insertional mutagenesis has been widely employed to

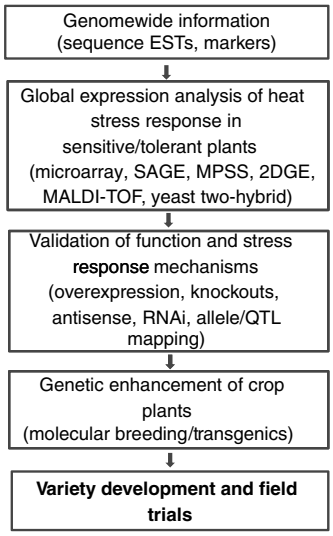

Figure 29.3 Different genomics and proteomics approaches used in understanding heat stress tolerance mechanism.

characterize abiotic stress-responsive genes, including those coding for *At HKT1, CBL1, OsRLK1, CIPK3, OSM1/SYP61,* and *HOS10.*

When expression profiling is undertaken in plant tissue, it is necessary to identify protein components to maximize the information that can be gleaned from expression profile. Analysis of proteome provides a direct link of the genome sequence to biological activity. Analysis of the proteome includes acquiring knowledge of the entire protein repertoire as well as other aspects, such as expression levels, post-

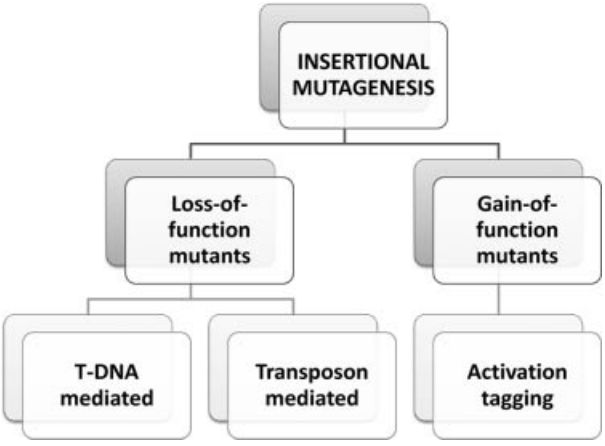

Figure 29.4 Insertional mutagenesis approaches for identifying genes responsible for heat tolerance.

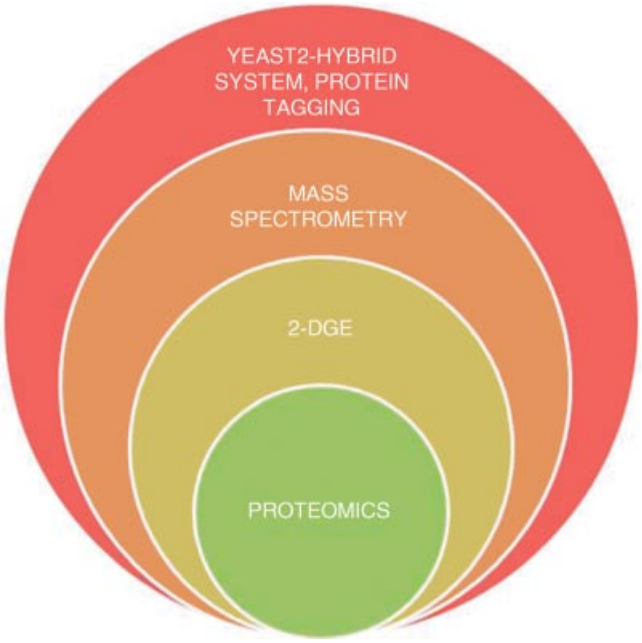

Figure 29.5 Major components involved in proteomics.

translational modifications, and interactions, to understand the cellular processes at the protein level.

Proteomics is a powerful approach to study changes in the proteome of plant system under various stresses including heat stress (see Figure 29.5). Techniques such as two-dimensional electrophoresis, Y2H (yeast two-hybrid) assay, and mass spectrometry offer opportunity to discover the various proteins involved in heat tolerance mechanism. Proteomic reference maps have been made for various crops including wheat [180] at various growth stages. Upregulation of various heat shock proteins and downregulation of several other proteins (e.g., proteins involved in starch metabolism) have been shown in wheat under heat stress during grain filling [102, 181]. Proteome approach has been successfully used to study the effect of heat shock on wheat grain quality using 2D gel electrophoresis and to identify protein markers that enable breeders to produce cultivars with desired characters under heat stress conditions [182].

Identifying novel genes and studying their expression patterns in response to heat stress will provide a molecular basis for improving heat tolerance in crops. Microarray analysis of gene expression has been used to investigate transcriptome changes in response to heat stress and combined stresses in several plant species [183, 184], including wheat [185]. Using Affymetrix Wheat Genome Array, it was found that ~11% (6560) of total probe sets were responsive to short and prolonged heat stress treatments in wheat and hence it was concluded that the differences in heat tolerance in different wheat genotypes may be associated with multiple processes and

mechanisms involving HSPs, transcription factors, and other stress-related genes [185]. Heat acclimation has little effects on gene expression under prolonged treatments, but it affects gene expression in wheat under short-term heat stress. The heat stress-responsive genes identified through micoarray will facilitate our understanding of molecular basis for heat tolerance in different wheat genotypes and future improvement of heat tolerance in wheat and other cereals.

Metabolite profiling has also been used to characterize stress responses to many abiotic stresses such as water deficit (dehydration and high salinity) and extreme temperature (cold and heat) for comprehensive analyses of the final steps of stress signal transduction pathways. A comparative metabolite analysis between *Arabidopsis* Columbia (Col-0) plants responding to heat shock and cold shock was carried out using GC-MS (gas chromatography–mass spectrometry) [186] and GC-TOF–MS (gas chromatography–time-of-flight mass spectrometry) [187]. The majority of metabolites produced in response to heat shock overlapped with those produced in response to cold shock. Moreover, these results suggested that a metabolic network of compatible solutes including proline, monosaccharides (glucose and fructose), galactinol, and raffinose has an important role in tolerance to temperature stress.

In summary, genetic engineering for improving plant heat stress tolerance is still at its infancy, and the success to date represents only a beginning. Advancements in marker technology and genetic engineering are expected to contribute significantly to the development of plants with tolerance to high temperatures in future. Future knowledge of tolerance components and the identification and cloning of responsible genes may allow transformation of plants with multiple genes and production of highly stress-tolerant transgenic plants. In addition, there is no report to date of any study testing the performance of transgenic plants under field stress conditions. Therefore, much more work is needed to gain a clearer understanding of the genetic, biochemical, and physiological basis of plant heat tolerance. To improve plant heat tolerance, alternative approaches to genetic means would include pretreatment of plants or seeds with heat stress or certain mineral or organic compounds. The success of such approaches, however, depends on plant species and genotypes and must be studied on a case-to-case basis.

29.7
Energy Crises during Heat Stress

Under heat stress, various events interfere with the energy production via photosynthesis and electron transport chain in mitochondria and hence lead to reduction in growth of plant. During light reactions, due to increase in leaf temperature, there is an increase in ATP production to meet the energy requirement of the cell. In dark reactions, rubisco activation is inhibited under heat stress, which leads to reduction in carbohydrate production due to interrupted C_3 cycle [188, 189]. Heat stress leads to oxidative stress and DNA damage, which induces the poly(ADP)-ribosylation of various nuclear proteins as their posttranslational modification. This modification is achieved by an enzyme named poly(ADP-ribose) polymerase (PARP), whose activity

is enhanced under various environmental stresses including high temperature. As NAD^+ is used as a substrate for this enzyme, an increase in the activity leads to a decrease in the pool of NAD^+. Since ATP molecules are required to resynthesize the depleted NAD^+, these reactions deplete the energy of the plant due to ATP overconsumption and enhance the production of ROS, which eventually leads to cell death [190]. Hence decreasing the poly(ADP)-ribosylation can prove an effective strategy for maintaining the plant energy homeostasis under adverse conditions such as high temperature. In short, heat tolerance in plants is a cost-intensive process and consumes considerable cellular energy to cope with adversaries such as a high temperature.

29.8 Conclusions and Outlook

Great variation exists within plant species in response to heat stress, which also varies with developmental stage. However, heat stress affects plant growth almost at every developmental stage of the plant, but for wheat, the grain-filling stage has been reported as the most susceptible. Membrane disruption, disarrangement of cell organelles, disturbance in leaf water relations, impaired photosynthesis due to changes in series of biochemical and photochemical reactions, production of ROS, and lipid peroxidation and hence generation of oxidative stress in plant cells are some of the adverse effects that are caused by heat stress. Various adaptive strategies have been reported in plants in response to heat stress. These include initiation of signaling cascade leading to profound changes in specific gene expression in which the role of Ca^{2+} is most important; enhanced expression of HSPs that act as molecular chaperones that play a role in preventing protein denaturation and removing denatured proteins and dissolving protein aggregates in poststress period to reduce toxicity. While adaptation to stress under natural conditions has some ecological advantages, the metabolic and energy costs may sometimes mask and limit its benefit to agriculture and result in yield penalty. Therefore, the improvement in abiotic stress tolerance in agricultural plants can be achieved, practically, by combining traditional and molecular breeding [191–193]. Conventional breeding strategies have shown some notable progress in the development of crop plants with improved heat tolerance, but the progress is slow due to inherent difficulties of selecting the desired genotypes. More emphasis should be given to identifying the molecular markers linked to component traits for heat tolerance so that these could be used for marker-assisted selection. A comprehensive understanding of the molecular, physiological, and biochemical basis of the mechanism of heat tolerance in some plant species has paved the way for genetically engineering the plants that will withstand the heat stress to produce better yield in heat stress environments.

Global warming is likely to increase the frequency of heat stress episodes and cause yield loss. There is need to be aware of this likelihood and take adaptive management and breeding steps to reduce the associated risks. These steps should improve further research to understand the mechanisms involved in heat stress tolerance and provide

breeders with selection tools to assist in the production of varieties that will tolerate heat stress. Among several wild species of wheat, *Aegilops speltoides* appears to have the highest level of heat tolerance. This species continues to grow without any adverse effect on pollen fertility even in the month of May in Punjab state of India, when the temperature is 40 plus. The authors' group is exploring the mechanism of heat tolerance in this species and attempting to transfer the trait to hexaploid wheat using tetraploid wheat *T. durum* as a bridging species.

References

1 Joshi, A.K., Mishra, B., Chatrath, R. *et al.* (2007) Wheat improvement in India: present status, emerging challenges and future prospects. *Euphytica*, **157**, 431–446.

2 Mitra, R. and Bhatia C.R. (2008) Bioenergetic cost of heat tolerance in wheat crop. *Curr. Sci.*, **94**, 1049–1053.

3 Fischer, R.A. and Byerlee, D.R. (1991) Trends of wheat production in the warmer areas: major issues and economic considerations, in *Wheat for Non-traditional, Warm Areas* (ed. D.A. Saunders), CIMMYT, Mexico, DF, pp. 3–27.

4 Barah, B.C. (2006) Changing pattern of wheat economy in India. *NAAS News*, **6**, 5–7.

5 Lobell, D.B. and Field, C.B. (2007) Global scale climate–crop yield relationships and impacts of recent warming. *Environ. Res. Lett.*, **2**, 1–7.

6 Lawlor, D.W. and Mitchell, R.A.C. (2000) Wheat, in *Climate Change and Global Crop Productivity* (eds K.R. Reddy and H.F. Hodges), CAB International, Wallingford, pp. 57–80.

7 Blum, A. (1986) The effect of heat stress on wheat leaf and ear photosynthesis. *J. Exp. Bot.*, **37**, 111–118.

8 Gusta, L.V. and Chen, T.H. (1987) The physiology of water and temperature stress, in *Wheat and Wheat Improvement. Agronomy*, vol. 13 (ed. E.G. Heyne), American Society of Agronomy, Inc., Madison, WI, pp. 115–150.

9 Levitt, J. (1980) Responses of plants to environmental stresses, in *Chilling, Freezing and High Temperature Stresses*, 2nd edn, Academic Press, New York, pp. 407–414.

10 Porter, J.R. (2005) Rising temperatures are likely to reduce crop yields. *Nature*, **436**, 174.

11 Schoffl, F., Prandl, R., and Reindl, A. (1999) Molecular responses to heat stress, in *Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants* (eds K. Shinozaki and K. Yamaguchi-Shinozaki), R.G. Landes Co., Austin, Texas, pp. 81–98.

12 Howarth, C.J. (2005) Genetic improvements of tolerance to high temperature, in *Abiotic Stresses: Plant Resistance Through Breeding and Molecular Approaches* (eds M. Ashraf and P.J.C. Harris), Howarth Press, Inc., New York, pp. 277–300.

13 Smertenko, A., Draber, P., Viklicky, V., and Opatrny, Z. (1997) Heat stress affects the organization of microtubules and cell division in *Nicotiana tabacum* cells. *Plant Cell Environ.*, **20**, 1534–1542.

14 Al-Khatib, K. and Paulsen, G.M. (1999) High-temperature effects on photosynthetic processes in temperate and tropical cereals. *Crop. Sci.*, **39**, 119–125.

15 Iba, K. (2002) Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. *Annu. Rev. Plant Biol.*, **53**, 225–245.

16 Feder, M.E. and Hoffman, G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.*, **61**, 243–282.

17 Momcilovic, I. and Ristic, Z. (2007) Expression of chloroplast protein synthesis elongation factor, EF-Tu, in two lines of maize with contrasting

tolerance to heat stress during early stages of plant development. *J. Plant Physiol.*, **164**, 90–99.
18 Wahid, A. (2007) Physiological implications of metabolites biosynthesis in net assimilation and heat stress tolerance of sugarcane sprouts. *J. Plant Res*, **120**, 219–228.
19 Siddique, K.H.M., Loss, S.P., Regan, K.L., and Jettner, R.L. (1999) Adaptation and seed yield of cool season grain legumes in Mediterranean environments of south-western Australia. *Aust. J. Agric. Res.*, **50**, 375–387.
20 Mariamma, M., Muthukumar, B., Veluthambi, K., and Gnanam, A. (1997) Effects of high temperature stress on the expression of low molecular weight heat shock proteins in rice leaves. *J. Plant Physiol.*, **151**, 763–765.
21 Monjardino, P., Smith, A.G., and Jones, R.J. (2005) Heat stress effects on protein accumulation of maize endosperm. *Crop Sci.*, **45**, 1203–1210.
22 Vollenweider, P. and Gunthardt-Goerg, M.S. (2005) Diagnosis of abiotic and biotic stress factors using the visible symptoms in foliage. *Environ. Pollut.*, **137**, 455–465.
23 Guilioni, L., Wery, J., and Lecoeur, J. (2003) High temperature and water deficit may reduce seed number in field pea purely by decreasing plant growth rate. *Funct. Plant Biol.*, **30**, 1151–1164.
24 Ferris, R., Ellis, R.H., Wheeler, T.R., and Hadley, P. (1998) Effect of high temperature stress at anthesis on grain yield and biomass of field grown crops of wheat. *Plant Cell Environ*, **34**, 67–78.
25 Stone, P.J. and Nicolas, M.E. (1994) Wheat cultivars vary widely in their responses of grain yield and quality to short periods of post-anthesis heat stress. *Aust. J. Plant Physiol.*, **21**, 887–900.
26 Anon, S., Fernandez, J.A., Franco, J.A. *et al.* (2004) Effects of water stress and night temperature preconditioning on water relations and morphological and anatomical changes of *Lotus creticus* plants. *Sci. Hortic.*, **101**, 333–342.
27 Karim, M.A., Fracheboud, Y., and Stamp, P. (1997) Heat tolerance of maize with reference of some physiological characteristics. *Ann. Bangladesh Agri.*, **7**, 27–33.
28 Zhang, J.H., Huang, W.D., Liu, Y.P., and Pan, Q.H. (2005) Effects of temperature acclimation pretreatment on the ultrastructure of mesophyll cells in young grape plants (*Vitis vinifera* L. cv. Jingxiu) under cross-temperature stresses. *J. Integr. Plant Biol.*, **47**, 959–970.
29 Guilioni, L., Wery, J., and Tardieu, F. (1997) Heat stress-induced abortion of buds and flowers in pea: is sensitivity linked to organ age or to relations between reproductive organs? *Ann. Bot.*, **80**, 159–168.
30 Young, L.W., Wilen, R.W., and Bonham-Smith, P.C. (2004) High temperature stress of *Brassica napus* during flowering reduces micro- and megagametophyte fertility, induces fruit abortion, and disrupts seed production. *J. Exp. Bot.*, **55**, 485–495.
31 Sato, S., Kamiyama, M., Iwata, T. *et al.* (2006) Moderate increase of mean daily temperature adversely affects fruit set of *Lycopersicon esculentum* by disrupting specific physiological processes in male reproductive development. *Ann. Bot.*, **97**, 731–738.
32 Perrotta, C., Treglia, A.S., Mita, G. *et al.* (1998) Analysis of mRNAs from ripening wheat seeds: the effect of high temperature. *J. Cereal Sci.*, **27**, 127–132.
33 Wardlaw, I.F., Blumenthal, C., Larroque, O., and Wrigley, C.W. (2002) Contrasting effects of chronic heat stress and heat shock on kernel weight and flour quality in wheat. *Funct. Plant Biol.*, **29**, 25–34.
34 Al-Khatib, K. and Paulsen, G.M. (1984) Mode of high temperature injury to wheat during grain development. *Plant Physiol.*, **61**, 363–368.
35 Jenner, C.F. (1994) Starch synthesis in the kernel of wheat under high temperature conditions. *Aust. J. Plant Physiol*, **21**, 791–806.
36 Keeling, P.L., Bacon, P.J., and Holt, D.C. (1993) Elevated temperature reduces starch deposition in wheat endosperm by reducing the activity of soluble starch synthase. *Planta*, **191**, 342–348.

37 Owen, P.C. (1971) Responses of a semi-dwarf wheat to temperatures representing a tropical dry season. II. Extreme temperatures. *Exp. Agric.*, **7**, 43–47.

38 Saini, H.S. and Aspinal, D. (1982) Abnormal sporogenesis in wheat (*Triticum aestivum* L.) induced by short periods of high temperature. *Ann. Bot.*, **49**, 835–846.

39 Rahman, M.S., Wilson, J.H., and Aitken, V. (1977) Determination of spikelet number in wheat. II. Effect of varying light level on ear development. *Aust. J. Agric. Res.*, **26**, 575–581.

40 Fischer, R.A. (1985) Number of kernels in wheat crops and the influence of solar radiation and temperature. *J. Agric. Sci.*, **105**, 447–461.

41 Mishra, S.P. and Mohapatra, P.K. (1987) Soluble carbohydrates and floret fertility in wheat in relation to population density stress. *Ann. Bot.*, **60**, 269–277.

42 Abbate, P.E., Andrade, F.H., and Culot, J.P. (1995) The effect of radiation and nitrogen on number of grains in wheat. *J. Agric. Sci.*, **124**, 351–360.

43 Demotes-Mainard, S. and Jeuffroy, M.H. (2004) Effects of nitrogen and radiation on dry matter and nitrogen accumulation in the spike of winter wheat. *Field Crops Res.*, **87**, 221–233.

44 Kirby, E.J.M. (1988) Analysis of leaf, stem and ear growth in wheat from terminal spikelet stage to anthesis. *Field Crops Res*, **18**, 127–140.

45 Spiertz, J.H.J., Hamer, R.J., Xu, H. *et al.* (2006) Heat stress in wheat (*Triticum aestivum* L.): effects on grain growth and quality traits. *Eur. J. Agronomy*, **25**, 89–95.

46 Machado, S. and Paulsen, G.M. (2001) Combined effects of drought and high temperature on water relations of wheat and sorghum. *Plant Soil*, **233**, 179–187.

47 Simoes-Araujo, J.L., Rumjanek, N.G., and Margis-Pinheiro, M. (2003) Small heat shock proteins genes are differentially expressed in distinct varieties of common bean. *Braz. J. Plant Physiol.*, **15**, 33–41.

48 Sairam, R.K. and Tyagi, A. (2004) Physiology and molecular biology of salinity stress tolerance in plants. *Curr. Sci.*, **86**, 407–421.

49 Sakamoto, A. and Murata, N. (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant Cell Environ.*, **25**, 163–171.

50 Wahid, A. and Close, T.J. (2007) Expression of dehydrins under heat stress and their relationship with water relations of sugarcane leaves. *Biol. Plant.*, **51**, 104–109.

51 Kinnersley, A.M. and Turano, F.J. (2000) Gamma aminobutyric acid (GABA) and plant responses to stress. *Crit. Rev. Plant Sci.*, **19**, 479–509.

52 Larcher, W. (1995) *Physiological Plant Ecology: Ecophysiology and Stress Physiology of Functional Groups*, 3rd edn, Springer, Berlin.

53 Wise, R.R., Olson, A.J., Schrader, S.M., and Sharkey, T.D. (2004) Electron transport is the functional limitation of photosynthesis in field-grown Pima cotton plants at high temperature. *Plant Cell Environ*, **27**, 717–724.

54 Mcdonald, G.K. and Paulsen, G.M. (1997) High temperature effects on photosynthesis and water relations of grain legumes. *Plant Soil*, **196**, 47–58.

55 De Ronde, J.A.D., Cress, W.A., Kruger, G.H.J. *et al.* (2004) Photosynthetic response of transgenic soybean plants containing an *Arabidopsis P5CR* gene, during heat and drought stress. *J. Plant Physiol.*, **61**, 1211–1244.

56 Yamane, Y., Kashino, Y., Koike, H., and Satoh, K. (1998) Effects of high temperatures on the photosynthetic systems in spinach: oxygen-evolving activities, fluorescence characteristics and the denaturation process. *Photosynth. Res.*, **57**, 51–59.

57 Sharkova, V.E. (2001) The effect of heat shock on the capacity of wheat plants to restore their photosynthetic electron transport after photoinhibition or repeated heating. *Russ. J. Plant Physiol.*, **48**, 793–797.

58 Wardlaw, J.F., Sofield, I., and Cartwright, P.M. (1980) Factors limiting the rate of dry matter accumulation in the grain

of wheat grown at high temperature. *Aust. J. Plant Physiol.*, **7**, 387–400.

59 Harding, S.A., Guikema, J.A., and Paulsen, G.M. (1990) Photosynthetic decline from high temperature stress during maturation of wheat. I. Interaction with senescence process. *Plant Physiol.*, **92**, 648–653.

60 Salvucci, M.E. and Crafts-Brandner, S.J. (2004) Relationship between the heat tolerance of photosynthesis and the thermal stability of Rubisco activase in plants from contrasting thermal environments. *Plant Physiol*, **134**, 1460–1470.

61 Todorov, D.T., Karanov, E.N., Smith, A.R., and Hall, M.A. (2003) Chlorophyllase activity and chlorophyll content in wild type and *eti 5* mutant of *Arabidopsis thaliana* subjected to low and high temperatures. *Biol. Plant.*, **46**, 633–636.

62 Kepova, K.D., Holzer, R., Stoilova, L.S., and Feller, U. (2005) Heat stress effects on ribulose-1,5-bisphosphate carboxylase/oxygenase, Rubisco binding protein and Rubisco activase in wheat leaves. *Biol. Plant.*, **49**, 521–525.

63 Chaitanya, K.V., Sundar, D., and Reddy, A.R. (2001) Mulberry leaf metabolism under high temperature stress. *Biol. Plant.*, **44**, 379–384.

64 Vu, J.C.V., Gesch, R.W., Pennanen, A.H. *et al.* (2001) Soybean photosynthesis, Rubisco and carbohydrate enzymes function at supra-optimal temperatures in elevated CO_2. *J. Plant Physiol.*, **158**, 295–307.

65 Morales, D., Rodriguez, P., Dellamico, J. *et al.* (2003) High-temperature preconditioning and thermal shock imposition affects water relations, gas exchange and root hydraulic conductivity in tomato. *Biol. Plant.*, **47**, 203–208.

66 Laing, W.A., Ogren, W.L., and Hageman, R.H. (1974) Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2, O_2 and ribulose 1,5-diphosphate carboxylase. *Plant Physiol.*, **54**, 678–685.

67 Monson, R.K., Stidham, M., Williams, G.J. *et al.* (1982) Temperature dependence of photosynthesis in *Agropyron smithii Rydb. Plant Physiol.*, **69**, 921–928.

68 Jordan, D.B. and Ogren, W.L. (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. Dependence on ribulosebisphosphate concentration, pH and temperature. *Planta.*, **161**, 308–313.

69 Nakamoto, H. and Hiyama, T. (1999) Heat-shock proteins and temperature stress, in *Handbook of Plant and Crop Stress*, Marcel Dekker, New York, pp. 399–416.

70 Stone, P. (2001) The effects of heat stress on cereal yield and quality, in *Crop Responses and Adaptation to Temperature Stress* (ed. A.S. Basra), Food Products Press, Binghamton, NY, pp. 243–291.

71 Yang, J., Sears, R.G., Gill, B.S., and Paulsen, G.M. (2002) Genotypic differences in utilization of assimilate sources during maturation of wheat under chronic heat and heat shock stresses. *Euphytica*, **125**, 179–188.

72 Blum, A., Klueva, N., and Nguyen, H.T. (2001) Wheat cellular thermotolerance is related to yield under heat stress. *Euphytica*, **117**, 117–123.

73 Blum, A. (1988) *Plant Breeding for Stress Environments*, CRC Press Inc., Boca Raton, Florida.

74 Blum, A. and Ebercon, A. (1981) Cell membrane stability as a measure of drought and heat tolerance in wheat. *Crop Sci.*, **21**, 43–47.

75 Shanahan, J.F., Edwards, I.B., Quick, J.S., and Fenwick, R.J. (1990) Membrane thermostability and heat tolerance of spring wheat. *Crop Sci.*, **30**, 247–25.

76 Maestri, E., Klueva, N., Perrotta, C. *et al.* (2002) Molecular genetics of heat tolerance and heat shock proteins in cereals. *Plant Mol. Biol.*, **48**, 667–681.

77 Davies, W.J. and Jones, H.G. (1991) *Abscisic Acid: Physiology and Biochemistry*, BIOS Scientific Publishers, Oxford, UK.

78 Xiong, L., Lee, H., Ishitani, M., and Zhu, J.K. (2002) Regulation of osmotic stress responsive gene expression by LOS6/ABA1 locus in *Arabidopsis. J. Biol. Chem.*, **277**, 8588–8596.

79 Pareek, A., Singla, S.L., and Grover, A. (1998) Proteins alterations associated

with salinity, desiccation, high and low temperature stresses and abscisic acid application in seedlings of Pusa 169, a high-yielding rice (*Oryza sativa* L.) cultivar. *Curr. Sci.*, **75**, 1023–1035.

80 Lee, G. and Vierling, E. (2000) A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat denatured protein. *Plant Physiol.*, **122**, 189–198.

81 Arshad, M. and Frankenberger, W.T.J. (2002) *Ethylene, Agricultural Sources and Applications*, Kluwer Academic/Plenum Publishers, New York.

82 Tan, C., Yu, Z.W., Yang, H.D., and Yu, S.W. (1988) Effect of high temperature on ethylene production in two plant tissues. *Acta Phytophysiol. Sin.*, **14**, 373–379.

83 Kawano, T., Sahashi, N., Takahashi, K. *et al.* (1998) Salicylic acid induces extracellular superoxide generation followed by an increase in cytosolic calcium ion in tobacco suspension culture: the earliest events in salicylic acid signal transduction. *Plant Cell Physiol.*, **39**, 721–730.

84 Wang, L.J. and Li, S.H. (2006) Thermotolerance and related antioxidant enzyme activities induced by heat acclimation and salicylic acid in grape (*Vitis vinifera* L.) leaves. *Plant Growth Regul.*, **48**, 137–144.

85 Horton, P. (2002) Crop Improvement Through Alteration in the Photosynthetic Membrane, ISB News Report. Virginia Tech., Blacksburg, VA.

86 Upadhyaya, A., Davis, T.D., Larsen, N.H. *et al.* (1990) Uniconazole-induced thermotolerance in soybean seedling root tissue. *Physiol. Plant.*, **79**, 78–84.

87 Davidson, J.E., Whyte, B., Bissinger, P.H., and Schiestl, R.H. (1996) Oxidative stress is involved in heat induced cell death in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci., USA*, **93**, 5116–5121.

88 Liu, X. and Huang, B. (2000) Heat stress injury in relation to membrane lipid peroxidation in creeping bent grass. *Crop Sci.*, **40**, 503–510.

89 Imlay, J.A., Chin, S.M., and Linn, S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro*. *Science*, **240**, 640–642.

90 Larson, J.S., Schuetz, T.J., and Kingston, R.E. (1988) Activation *in vitro* of sequence-specific DNA binding by a human regulatory factor. *Nature*, **335**, 372–375.

91 Zhou, R.G., Fan, Z.H., Li, X.Z. *et al.* (1995) The effect of heat acclimation on membrane thermostability and relative enzyme activity. *Acta Agron. Sin.*, **21**, 568–572.

92 Dash, S. and Mohanty, N. (2002) Response of seedlings to heat-stress in cultivars of wheat: growth temperature-dependent differential modulation of photosystem 1 and 2 activity and foliar antioxidant defense capacity. *J. Plant Physiol.*, **159**, 49–59.

93 Hoekstra, F.A., Golovina, E.A., and Buitink, J. (2001) Mechanisms of plant desiccation tolerance. *Trends Plant Sci.*, **6**, 431–438.

94 Prasinos, C., Krampis, K., Samakovli, D., and Hatzopoulos, P. (2005) Tight regulation of expression of two *Arabidopsis* cytosolic *Hsp90* genes during embryo development. *J. Exp. Bot.*, **56**, 633–644.

95 Vierling, E. (1991) The role of heat shock proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*, **42**, 579–620.

96 Bowen, J., Michael, L.Y., Plummer, K.I.M., and Ferguson, I.A.N. (2002) The heat shock response is involved in thermotolerance in suspension-cultured apple fruit cells. *J. Plant Physiol.*, **159**, 599–606.

97 Jinn, T.L., Yeh, Y.C., Chen, Y.M., and Lin, C.Y. (1989) Stabilization of soluble proteins *in vitro* by heat shock proteins enriched ammonium sulphate fraction from soybean seedlings. *Plant Cell Physiol.*, **30**, 463–469.

98 Burke, J.J. (2001) Identification of genetic diversity and mutations in higher plant acquired thermotolerance. *Physiol. Plant.*, **112**, 167–170.

99 O'Mahony, P. and Burke, J. (2000) A ditelosomic line of "Chinese Spring" wheat with augmented acquired thermotolerance. *Plant Sci.*, **158**, 147–154.

100 O'Mahony, P., Burke, J.J., and Oliver, M.J. (2000) Identification of acquired thermotolerance deficiency within the ditelosomic series of "Chinese Spring" wheat. *Plant Physiol. Biochem.*, **38**, 243–252.

101 Ortiz, C. and Cardemil, L. (2001) Heat-shock responses in two leguminous plants: a comparative study. *J. Exp. Bot.*, **52**, 1711–1719.

102 Majoul, T., Bancel, E., Triboi, E. *et al.* (2004) Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from non-prolamins fraction. *Proteomics*, **4**, 505–513.

103 Thomashow, M.F. (1998) Role of cold responsive genes in plant freezing tolerance. *Plant Physiol.*, **118**, 1–7.

104 Vinocur, B. and Altman, A. (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr. Opin. Biotechnol.*, **16**, 123–132.

105 Bohnert, H.J., Gong, Q., Li, P., and Ma, S. (2006) Unravelling abiotic stress tolerance mechanisms: getting genomics going. *Curr. Opin. Plant Biol.*, **9**, 180–188.

106 Queitsch, C., Hong, S.W., Vierling, E., and Lindquest, S. (2000) Heat shock protein 101 plays a crucial role in thermotolerance in *Arabidopsis. Plant Cell*, **12**, 479–492.

107 Sung, D.Y., Kaplan, F., Lee, K.J., and Guy, C.L. (2003) Acquired tolerance to temperature extremes. *Trends Plant Sci.*, **8**, 179–187.

108 Diamant, S., Eliahu, N., Rosenthal, D., and Goloubinoff, P. (2001) Chemical chaperones regulate molecular chaperones *in vitro* and in cells under combined salt and heat stresses. *J. Biol. Chem.*, **276**, 39586–39591.

109 Wang, W., Vinocur, B., Shoseyov, O., and Altman, A. (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci.*, **9**, 244–252.

110 Panchuk, I.I., Volkov, R.A., and Scoffl, F. (2002) Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in *Arabidopsis. Plant Physiol.*, **129**, 838–853.

111 Klueva, N.Y., Maestri, E., Marmiroli, N., and Nguyen, H.T. (2001) *Mechanisms of Thermotolerance in Crops* (ed. A.S. Basra), Food Products Press, Binghamton, NY, pp. 177–217.

112 Burke, J.J., O'Mahony, P.J., and Oliver, M.J. (2000) Isolation of *Arabidopsis* mutants lacking components of acquired themotolerance. *Plant Physiol.*, **123**, 575–588.

113 Hong, S.W. and Vierling, E. (2000) Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. *Proc. Natl. Acad. Sci. USA*, **97**, 4392–4397.

114 Heckathorn, S.A., Downs, C.A., and Coleman, J.S. (1998) Nuclear-encoded chloroplast proteins accumulated in the cytosol during severe heat stress. *Int. J. Plant Sci.*, **159**, 39–45.

115 Nollen, E.A.A. and Morimoto, R.I. (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing "heat shock" proteins. *J. Cell Sci.*, **115**, 2809–2816.

116 Arrigo, A.P. (1998) Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. *Biol. Chem.*, **379**, 19–26.

117 Yang, K.A., Lim, C.J., Hong, J.K. *et al.* (2006) Identification of cell wall genes modified by a permissive high temperature in Chinese cabbage. *Plant Sci.*, **171**, 175–182.

118 Yost, H.J. and Lindquist, S. (1986) RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell*, **45**, 185–193.

119 Midmore, D.J., Cartwright, P.M., and Fischer, R.A. (1984) Wheat in tropical environments. II. Crop growth and grain yield. *Field Crops Res.*, **8**, 207–227.

120 Rawson, H.N. (1986) High temperature-tolerant wheat: a description of variation and a search for some limitations to productivity. *Field Crops Res.*, **14**, 197–212.

121 Al-Khatib, K., and Paulsen, G.M. (1990) Photosynthesis and productivity during high temperature stress of wheat cultivars from major world regions. *Crop Sci*, **30**, 1127–1132.

122 Reynolds, M.P., Balota, M., Delgado, M.I.B. *et al.* (1994) Physiological and morphological traits associated with spring wheat yield under hot, irrigated conditions. *Aust. J. Plant Physiol.*, **21**, 717–30.

123 Edhaie, B. and Waines, J.G. (1992) Heat resistance in wild *Triticum* and *Aegilops*. *J. Genet. Breed.*, **46**, 221–228.

124 Chinnusamy, V., Schumaker, K., and Zhu, J.K. (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signaling in plants. *J. Exp. Bot.*, **55**, 225–236.

125 Suzuki, N. and Mittler, R. (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiol. Plant.*, **126**, 45–51.

126 Plieth, C. (1999) Temperature sensing by plants: calcium-permeable channels as primary sensors: a model. *J. Membr. Biol.*, **172**, 121–127.

127 Gong, M., Chen, S.N., Song, Y.Q., and Li, Z.G. (1997) Effect of calcium and calmodulin on intrinsic heat tolerance in relation to antioxidant systems in maize seedlings. *Aust. J. Plant Physiol.*, **24**, 371–379.

128 Webb, A.A.R., Mcainsh, M.R., Taylor, J.E., and Hetherington, A.M. (1996) Calcium ions as intercellular second messengers in higher plants. *Adv. Bot. Res.*, **22**, 45–96.

129 Wang, J.B. and Li, R.Q. (1999) Changes of Ca^{2+} distribution in mesophyll cells of pepper under heat stress. *Acta Hortic. Sin.*, **26**, 57–58.

130 Joyce, S.M., Cassells, A.C., and Mohan, J.S. (2003) Stress and aberrant phenotypes *in vitro* culture. *Plant Cell Tissue Organ Cult.*, **74**, 103–121.

131 Warren, G.F. (1998) Spectacular increases in crop yields in the twentieth century. *Weed Technol.*, **12**, 752–760.

132 Setimela, P.S., Andrews, D.J., Partridge, J., and Eskridge, K.M. (2005) Screening sorghum seedlings for heat tolerance using a laboratory method. *Eur. J. Agron.*, **23**, 103–107.

133 Amani, J., Fischer, R.A., and Reynolds, M.P. (1996) Canopy temperature depression association with yield of irrigated spring wheat cultivars in a hot climate. *J. Agron. Crop Sci.*, **176**, 119–129.

134 Hede, A., Skovmand, B., Reynolds, M.P. *et al.* (1999) Evaluating genetic diversity for heat tolerance in Mexican wheat landraces. *Genet. Resour. Crop Evol.*, **46**, 37–45.

135 Reynolds, M.P. and Borlaug, N.E. (2006) International collaborative wheat improvement: impacts and future prospects. *J. Agric. Sci.*, **144**, 3–17.

136 Ortiz, R., Trethowan, R., Ortiz Ferrara, G.F. *et al.* (2007) High yield potential, shuttle breeding, genetic diversity and new international wheat improvement strategy. *Euphytica*, **57**, 365–384.

137 Scott, J.W., Volin, R.B., Bryan, H.H., and Olson, S.M. (1986) Use of hybrids to develop heat tolerant tomato cultivars. *Proc. Fla. State Hortic. Soc.*, **99**, 311–315.

138 Scott, J.W., Olson, S.M., Howe, T.K. *et al.* (1995) "Equinox" heat-tolerant hybrid tomato. *Hort. Sci.*, **30**, 647–648.

139 Roff, D.A. (1997) *Evolutionary Quantitative Genetics*, Chapman & Hall, New York, NY.

140 Shah, M.M., Gill, K.S., Baenziger, P.S. *et al.* (1999) Molecular mapping of loci for agronomic traits on chromosome 3A of bread wheat. *Crop Sci.*, **39**, 1728–1732.

141 Roder, M.S., Korzun, V., Wendehake, K. *et al.* (1998) A microsatellite map of wheat. *Genetics*, **149**, 2007–2023.

142 Penner, G.A., Clarke, J., Bezte, L.J., and Leisle, D. (1995) Identification of RAPD markers linked to a gene governing cadmium uptake in durum wheat. *Theor. Appl. Genet.*, **38**, 543–540.

143 Plaschke, J. and Roder, M.S. (1995) Detection of genetic diversity in closely related bread wheats using microsatellite markers. *Theor. Appl. Genet.*, **91**, 1001–1007.

144 Roder, M.S., Plaschke, J., König, S.U. *et al.* (1995) Abundance, variability and chromosomal location of microsatellite loci in wheat. *Mol. Gen. Genet.*, **246**, 327–333.

145 Korzun, V., Roder, M.S., Wendehake, K. *et al.* (1999) Integration of dinucleotide microsatellites from hexaploid bread wheat into a genetic linkage map of

durum wheat. *Theor. Appl. Genet.*, **98**, 1202–1207.

146 Stephenson, P., Bryan, G., Kirby, J. *et al.* (1998) Fifty new microsatellite loci for wheat genetic map. *Theor. Appl. Genet.*, **97**, 946–949.

147 Korzun, V., Borner, A., Wordland, A.J. *et al.* (1997) Application of microsatellite markers to distinguish intervarietal chromosome substitution lines of wheat (*Triticum aestivum* L.). *Euphytica*, **95**, 147–155.

148 Korzun, V., Roder, M.S., Wordland, A.J., and Borner, A. (1997) Mapping of the dwarfing (*Rht12*) and vernalisation response (*Vrn1*) genes in wheat by using RFLP and microsatellite markers. *Plant Breed.*, **116**, 227–232.

149 Fahima, T., Roder, M.S., Grama, A., and Nevo, A. (1998) Microsatellite DNA polymorphism divergence in *Triticum dicoccoides* accessions highly resistant to yellow rust. *Theor. Appl. Genet.*, **91**, 187–195.

150 Prasad, M., Warshney, R.K., Kumar, A. *et al.* (1999) A microsatellite marker associated with a QTL for grain protein content on chromosome arm 2DL of bread wheat. *Theor. Appl. Genet.*, **99**, 341–345.

151 Roy, J.K., Prasad, M., Varshney, R.K. *et al.* (1999) Identification of a microsatellite on chromosomes 6B and a STS on 7D of bread wheat showing an association with preharvest sprouting tolerance. *Theor. Appl. Genet.*, **99**, 336–340.

152 Grover, A. (2002) Molecular biology of stress responses. *Cell Stress Chap.*, **7**, 1–5.

153 Wahid, A., Gelani, S., Ashraf, M., and Foolad, M.R. (2007) Heat tolerance in plants: an overview. *Environ. Exp. Bot.*, **61**, 199–223.

154 Czarnecka, E., Key, J.L., and Gurley, W.B. (1989) Regulatory domains of the Gmhsp 17.5-E heat shock promoter of soybean: a mutational analysis. *Mol. Cell Biol.*, **9**, 3457–3463.

155 Collard, B.C.Y. and Mackill, D.J. (2008) Marker assisted selection: an approach for precision plant breeding in the twenty-first century. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **363**, 557–572.

156 Dita, M.A., Rispail, N., Prats, E. *et al.* (2006) Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. *Euphytica.*, **147**, 1–24.

157 Grover, A., Agarwal, M., Katiyar-Agarwal, S. *et al.* (2000) Production of high temperature tolerant transgenic plants through manipulation of photosynthetic membrane lipids. *Curr. Sci.*, **79**, 557–559.

158 Burke, J.J. and Chen, J. (2006) Changes in cellular and molecular processes in plant adaptation to heat stress, in *Plant–Environment Interactions* (ed. B. Huang), CRC Press, pp. 27–46.

159 Grover, A., Pareek, A., Singla, S.L. *et al.* (1998) Engineering crops for tolerance against abiotic stresses through gene manipulation. *Curr. Sci.*, **75**, 689–696.

160 Scharf, K.D., Siddique, M., and Vierling, E. (2001) The expanding family of *Arabidopsis thaliana* small heat stress proteins and a new family of proteins containing alpha-crystallin domains (Acd proteins). *Cell Stress Chap.*, **6**, 225–237.

161 Agarwal, M., Sarkar, N., and Grover, A. (2003) Low molecular weight heat shock proteins in plants. *J. Plant Biol.*, **30**, 141–149.

162 Grover, A., Aggarwal, P.K., Kapoor, A. *et al.* (2003) Addressing abiotic stresses in agriculture through transgenic technology. *Curr. Sci.*, **84**, 355–367.

163 Yeh, C.H., Chen, Y.M., and Lin, C.Y. (2002) Functional regions of rice heat shock protein, Oshsp16.9, required for conferring thermotolerance in *Escherichia coli*. *Plant Physiol.*, **128**, 661–668.

164 Hong, S.W. and Vierling, E. (2001) Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. *Plant J.*, **27**, 25–35.

165 Nieto-Sotelo, J., Martınez, L.M., Ponce, G. *et al.* (2002) Maize HSP101 plays important roles in both induced and basal thermotolerance and primary root growth. *Plant Cell*, **14**, 1621–1633.

166 Wu, C. (1995) Heat shock transcription factors: structure and regulation. *Annu. Rev. Cell Dev. Biol.*, **11**, 441–469.

167 Gepstein, S., Grover, A., and Blumwald, E. (2005) Producing biopharmaceuticals in the desert: building an abiotic stress tolerance in plants for salt, heat and drought, in *Modern Biopharmaceuticals* (eds J. Knablein and R.H. Muller), Wiley-VCH Verlag GmbH, Weinheim, pp. 967–994.

168 Yang, X., Liang, Z., and Lu, C. (2005) Genetic engineering of the biosynthesis of glycinebetaine enhances photosynthesis against high temperature stress in transgenic tobacco plants. *Plant Physiol*, **138**, 2299–2309.

169 Wang, G., Hui, Z., Li, F. *et al.* (2010) Improvement of heat and drought photosynthetic tolerance in wheat by overaccumulation of glycinebetaine. *Plant Biotechnol. Rep.*, **4**, 213–222.

170 Murakami, Y., Tsuyama, M., Kobayashi, Y., Kodama, H., and Iba, K. (2000) Trienoic fatty acids and plant tolerance of high temperature. *Science*, **287**, 476–479.

171 Havaux, M. (1998) Carotenoids as membrane stabilizers in chloroplasts. *Trends Plant Sci.*, **3**, 147–151.

172 Shi, W.M., Muramoto, Y., Ueda, A., and Takabe, T. (2001) Cloning of peroxisomal ascorbate peroxidase gene from barley and enhanced thermotolerance by overexpressing in *Arabidopsis thaliana*. *Gene*, **273**, 23–27.

173 Tang, L., Kwon, S.Y., Kim, S.H. *et al.* (2006) Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. *Plant Cell Rep.*, **25**, 1380–1386.

174 Fu, J., Momocilovic, I., Clemente, T.E. *et al.* (2008) Heterologous expression of a plastid EF-Tu reduces protein thermal aggregation and enhances CO_2 fixation in wheat (*Triticum aestivum*) following heat stress. *Plant Mol. Biol.*, **68**, 277–288.

175 Lee, J.H., Hiibel, A., and Schoffl, F. (1995) Derepression of the activity of genetically engineered heat shock factor causes constitutive synthesis of beat *shock* proteins and increased thermal tolerance in transgenic *Arabidopsis*. *Plant J.*, **8**, 603–612.

176 Malik, M.K., Slovin, J.P., Hwang, C.H., and Zimmerman, J.L. (1999) Modified expression of a carrot small heat shock protein gene, *hsp17.7*, results in increased or decreased thermotolerance. *Plant J.*, **20**, 89–99.

177 Kodama, H., Hamada, T., Horiguchi, G. *et al.* (1994) Genetic enhancement of cold tolerance by expression of a gene for chloroplast ω-3 fatty acid desaturase in transgenic tobacco. *Plant Physiol.*, **105**, 601–605.

178 Urano, K., Kurihara, Y., Seki, M., and Shinozaki, K. (2010) "Omics" analyses of regulatory networks in plant abiotic stress responses. *Curr. Opin. Plant Biol.*, **13**, 132–138.

179 Zhu, B., Ye, C., Lti, H. *et al.* (2006) Identification and characterization of a novel heat shock transcription factor gene, GmHsfAl, in soybeans (*Glycine max*). *J. Plant Res.*, **119**, 247–256.

180 Vensel, W.H., Tanka, C.K., Cai, N. *et al.* (2005) Developmental changes in the metabolic protein profiles of wheat endosperm. *Proteomics*, **5**, 1594–1611.

181 Majoul, T., Bancel, E., Triboi, E. *et al.* (2003) Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from total endosperm. *Proteomics*, **3**, 175–183.

182 Skylas, D.J., Cordwell, S.J., Hains, P.G. *et al.* (2002) Heat shock of wheat during grain filling: characterisation of proteins associated with heat-tolerance using a proteome approach. *J. Cereal Sci.*, **35**, 175–188.

183 Rizhsky, L., Liang, H., Shuman, J. *et al.* (2004) When defense pathways collide: the response of *Arabidopsis* to a combination of drought and heat stress. *Plant Physiol.*, **134**, 1683–1696.

184 Oshino, T., Abiko, M., Saito, R. *et al.* (2007) Premature progression of anther early developmental programs accompanied by comprehensive alterations in transcription during high-temperature injury in barley plants. *Mol. Genet. Genomics*, **278**, 31–42.

185 Qin, D., Wu, H., Peng, H. *et al.* (2008) Heat stress-responsive transcriptome analysis in heat susceptible and tolerant wheat (*Triticum aestivum* L.) by using wheat genome array. *BMC Genomics*, **9**, 432.

186 Kaplan, F., Kopka, J., Haskell, D.W. *et al.* (2004) Exploring the temperature-stress metabolome of *Arabidopsis*. *Plant Physiol.*, **136**, 4159–4168.

187 Wienkoop, S., Morgenthal, K., Wolschin, F. *et al.* (2008) Integration of metabolomic and proteomic phenotypes: analysis of data covariance dissects starch and RFO metabolism from low and high temperature compensation response in *Arabidopsis thaliana*. *Mol. Cell Proteomics*, **7**, 1725–1736.

188 Crafts-Brandner, S.J. and Salvucci, M.E. (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. *Proc. Natl. Acad. Sci. USA*, **97**, 13430–13435.

189 Dubey, R.S. (2005) Photosynthesis in plants under stressful conditions, in *Handbook of Photosynthesis* (ed. M. Pessarakli), CRC Press, Boca Raton, Florida, pp. 717–737.

190 De Block, M., Verduyn, C., De Brouwer, D., and Cornelissen, M. (2005) Poly(ADPribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. *Plant J.*, **41**, 95–106.

191 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. *et al.* (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.*, **17**, 287–291.

192 Dunwell, J.M. (2000) Transgenic approaches to crop improvement. *J. Exp. Bot.*, **51**, 487–496.

193 Wang, W.X., Vinocur, B., Shoseyov, O., and Altman, A. (2001) Biotechnology of plant osmotic stress tolerance: physiological and molecular considerations. *Acta Hort.*, **560**, 285–292.

30
Wheat and Rice Crops: "Omics" Approaches for Abiotic Stress Tolerance

Suja George, Ajay Parida, and Monkombu S. Swaminathan

Abiotic stresses are the principal causes of crop failure, reducing average yields of most major crops by more than 50%. Rice and wheat are the two most important food crops in the world, together occupying about 28% of all crop area. A considerable amount of crop biomass for which genetic potential exists in the present-day cultivars in wheat and rice is not harvested under field conditions, primarily because of the sensitivity of these crops to various stresses. To meet human needs by 2050, grain production must increase at an annual rate of 2% on an area of land over what is being produced at present. Modern biotechnology has a lot to offer in the field of crop improvement in the present scenario both in understanding the mechanisms of stress and stress tolerance in plants and in developing crop plants better equipped for harsher environmental conditions. A great deal of research has been carried out in the recent past in the field of plant abiotic stress tolerance encompassing genomics, transcriptomics, proteomics, and metabolomics. These "omic" technologies investigate different facets of a given scientific issue such as abiotic stress tolerance, but complement each other. Integration of phenotypic, genetic, transcriptomic, proteomic, and metabolomic data will enable accurate and detailed gene network reconstruction. This chapter discusses the recent "omic" studies in wheat and rice in the field of abiotic stress tolerance.

30.1
Introduction

Abiotic stresses such as drought, high salinity, low and high temperature, submergence, and so on are frequently encountered by plants in both natural and agricultural systems. In many cases, several classes of abiotic stress challenge plants in combination. For example, high temperatures and scarcity of water are commonly encountered in periods of drought and can be exacerbated by mineral toxicities that constrain root growth. Abiotic stresses are the principal causes of crop failure, reducing average yields of most major crops by more than 50% [1].

Rice (*Oryza sativa*) is one of the most important food crops in the world with almost half of the world's population depending on it as their staple food. More than 90% of

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the world's rice is grown and consumed in Asia, which is home to 60% of the Earth's population. Rice accounts for 35–75% of the calories consumed by more than 3 billion Asians. It is grown on about 154 million hectares annually or on about 11% of the world's cultivated land. Rice is cultivated in 42 million hectares (mha) under four major ecosystems, namely, irrigated (19 mha), rain-fed lowland (14 mha), flood-prone (3 mha), and rain-fed upland (6 mha) ecosystems. A considerable amount of rice biomass for which genetic potential exists in the present-day cultivars is not harvested under field conditions, primarily because of the sensitivity of this crop to various stresses [2].

Wheat (*Triticum aestivum* L.) was the first domesticated crop and is the youngest polyploid species among the agricultural crops. Together with rice and maize, wheat provides 60% of the calories and proteins for our daily life. Unlike rice and maize, which prefer tropical environments, wheat occupies 17% of all crop area (in 2002, 210 million hectares). The trade value of wheat exceeds that of any other cereal species, including rice and maize ($31 billion of world trade in 2001 versus $13 and $19 billion for rice and maize, FAOSTAT database: http://apps.fao.org/default.jsp). Wheat cultivators lose about 25% annually due to various biotic (pests) and abiotic stresses [3].

The world population is continuing to rise, but the gains in agricultural output provided by the Green Revolution have reached a plateau by now [4]. Many explanations have been offered for this, including deteriorating irrigation infrastructure, soil degradation, stagnant technology for rain-fed farms, and the technological fatigue being reached on irrigated farms. Adverse regional climate changes caused by the combined effects of atmospheric brown clouds (ABC) and greenhouse gases (GHG) add to this crisis. To meet human needs by 2050, grain production must increase at an annual rate of 2% on an area of land over what is being produced at present [2]. Modern biotechnology has a lot to offer in the area of crop improvement in the present scenario both in understanding the mechanisms of stress and stress tolerance in plants and in developing crop plants better equipped for harsher environmental conditions.

Since 2000, plant science has moved forward into the stage of postgenomics. A great deal of research has been carried out in the recent past in the field of plant abiotic stress tolerance encompassing genomics, transcriptomics, proteomics, and metabolomics. The availability of whole genome sequences, microarrays, micro-RNA libraries, and so on has opened up research and result avenues that did not exist a decade ago. Completed in 2003, the Human Genome Project was a 13-year project. However, with the next-generation sequencing technologies from companies such as Roche, Illumina, and Applied Biosystems, sequencing of the whole genome of higher organisms is being done with unprecedented speed. Researchers all over the world have been enthusiastically exploiting the latest technologies and bioinformatics tools to provide better insights into various facets of abiotic stress tolerance.

This chapter explores the progress of research in the field of genomics, transcriptomics, proteomics, and metabolomics in wheat and rice, the most important cereal crops, in the recent past.

30.2 Genomics

Major genomics initiatives have generated valuable data for the elucidation of the expressed portion of the genomes of higher plants. The genome sequencing of *Arabidopsis thaliana* was completed in 2000 (The Arabidopsis Initiative), while the finished sequence for rice was published in 2005 (project IRGS). The relatively small genome size of these model organisms was a key element in their selection as the first plant genomes to be sequenced with extensive coverage. On the other hand, wheat is an allohexaploid having three homologous genomes. These genomes have been designated as A, B, and D, with the coding regions of the homologous genes sharing more than 90% homology. Wheat genome is one of the largest among crop species with a haploid size of 16.7 billion bp [5], which is 110 and 40 times larger than *Arabidopsis* and rice, respectively [6]. The large size, combined with the high percentage (over 80%) of repetitive noncoding DNA, presents a major challenge for comprehensive sequencing of the wheat genome. Although common wheat genetic maps with molecular markers and cytological maps with deletion mutants of the chromosome segments have been constructed, the number of mapped DNA markers for both map-based cloning and anchoring of the genome positions remains restricted [7].

In 2005, the International Wheat Genome Sequencing Consortium (IWGSC) was created with the purpose of sequencing the complete genome of bread wheat. In the mean time, a significant insight into the expressed portion of the wheat genome has been gained through large-scale generation and analysis of ESTs. cDNA libraries prepared from different tissues exposed to various stress conditions and developmental stages are valuable tools to obtain the expressed and stress-regulated portion of the genome.

To identify genes involved in cold acclimation and associated stresses, a large-scale EST sequencing approach was undertaken by the Functional Genomics of Abiotic Stress (FGAS) project [8]. As part of this project, 73 521 quality-filtered ESTs were generated from 11 cDNA libraries constructed from wheat plants exposed to various abiotic stresses and at different developmental stages. In addition, 196 041 ESTs for which trace files were available from the National Science Foundation wheat EST sequencing program and DuPont were also quality filtered and used in the analysis. Assembly of the resolved ESTs generated a 75 488 unique sequence set (31 580 contigs and 43 908 singletons/singlets). Digital expression analyses indicated that the FGAS data set is enriched in stress-regulated genes. Over 43% of the unique sequence set was annotated and classified into functional categories according to Gene Ontology.

In a similar study [7], a comprehensive collection of ESTs was prepared from various tissues that develop during the wheat life cycle and from tissues subjected to stress. The study also examined their expression profiles *in silico*. By grouping the ESTs of recombinant clones randomly selected from the full-length cDNA library, the researchers were able to sequence 6162 independent clones with high accuracy. About 10% of the clones were found to be wheat unique genes, without any counterparts within the DNA database. Wheat clones that showed high homology

to those of rice were selected and their expression patterns in various tissues throughout the wheat life cycle and in response to abiotic stress treatments were investigated. Their results showed each clone's expression in various tissues and stress treatments and its variability in wheat and rice as a result of their diversification.

Comparative genomics is a powerful tool for investigating plant evolution at the whole genome level. In a comparative analysis of wheat and rice genomes, 4485 expressed sequence tags that were physically mapped in wheat chromosome bins were compared with the public rice genome sequence data using BLAST [9]. This rice genome view of homologous wheat genome locations based on comparative sequence analysis revealed numerous chromosomal rearrangements between the two species over the course of evolution.

In the case of rice, the availability of the whole genome has made it possible to do genome-wide analysis of specific classes of genes. Most of these analyses are quite exhaustive, including identification of all the genes in a specific class, transcript profiling, phylogenetic analysis, regrouping based on the phylogenetic analysis or the presence or absence of protein domains, identification of splice variants and intronless variants, expression in transgenic systems, proposing new functions, and so on. These studies often present a comprehensive view of the specific class of genes. There are many reports of genome-wide identification and characterization of genes functioning in abiotic stress tolerance in rice.

Genome-wide analysis resulted in identification of 79 glutathione S-transferases (GSTs) genes in the rice genome [10]. A phylogenetic analysis grouped the GST proteins into seven classes. Sequence analysis together with the organization of putative motifs indicated the potential diverse functions of GST gene family members in rice. The authors proposed that tandem gene duplications have contributed a major role in expansion of this gene family. Microarray data analysis revealed tissue/organ- and developmental stage-specific expression patterns of several rice GST genes. At least 31 GST genes showed response to plant hormones auxin and cytokinin. Furthermore, expression analysis showed the differential expression of quite a large number of GST genes during various abiotic stress (20), arsenate stress (32), and biotic stress (48) conditions. Many of the GST genes were commonly regulated by developmental processes, hormones, and abiotic and biotic stresses. The transcript profiling suggested overlapping and specific role(s) of GSTs during various stages of development in rice. The study also provides evidence for the role of GSTs in mediating crosstalk between various stress and hormone response pathways [10].

A similar whole-genome analysis revealed 103 genes encoding WRKY transcription factors in rice. Among them, the majority of rice WRKY genes (77.7%) were located in duplicated regions; 45.6% of WRKY genes were fragmentally duplicated and 35% of them were tandemly duplicated. These results suggested that genome duplications might be regarded as a major mechanism for expansion of this family in the rice genome. Under abiotic (cold, drought, and salinity) stresses and various phytohormone treatments, 54 WRKY genes exhibited significant differences in their transcript abundance; among them three genes were expressed only under stressed conditions. Among the stress-inducible genes, 13 genes were regulated only by

abiotic stresses, another set of 13 genes were responsive to only phytohormone treatments, and the remaining 28 genes were regulated by both factors, suggesting an interaction between abiotic stress and hormone signaling [11].

DREB2s (dehydration-responsive element binding protein 2s) are transcription factors that interact with a *cis*-acting DRE (dehydration-responsive element)/CRT (C-repeat) sequence and activate the expression of downstream genes involved in water and heat shock stress responses and tolerance. A study analyzed all five DREB2-type genes in rice (OsDREB2s: OsDREB2A, OsDREB2B, OsDREB2C, OsDREB2E, and OsABI4) to determine which of them contribute to plant stress responses. The expression patterns of these genes under abiotic stress conditions were studied and the subcellular localization and transcriptional activation activity of their translational products in protoplasts were examined. Only OsDREB2A and OsDREB2B showed abiotic stress-inducible gene expression. In addition, OsDREB2B showed nuclear-specific localization and the highest transactivation activity. OsDREB2B has functional and nonfunctional forms of its transcript similar to its orthologues in the grass family, and the functional form of its transcript was markedly increased during stress conditions. The splicing mechanism of OsDREB2B was analyzed with transgenic rice that expressed the nonfunctional transcript, which revealed that the nonfunctional form is not a precursor of the functional form, indicating that stress-inducible alternative splicing of pre-mRNA is an important mechanism for the regulation of OsDREB2B. Transgenic *Arabidopsis* plants overexpressing OsDREB2B showed enhanced expression of DREB2A target genes and improved drought and heat- shock stress tolerance. These results pointed out the key role of OsDREB2B in stress-responsive gene expression in rice [12]. Another database search identified 29 C3HC4-type RING finger family genes in rice. A comprehensive expression analysis of these genes has been performed using microarray data obtained from 27 tissues or organs of three rice genotypes, Minghui 63, Zhenshan 97, and Shanyou 63. Expression analysis of C3HC4-type RING finger genes under abiotic stresses suggested that 12 genes are differentially regulated by hormones or stress in rice seedlings [13].

Heat shock proteins (Hsps) constitute an important component in the heat shock response of all living systems. Among the various plant Hsps (i.e., Hsp100, Hsp90, Hsp70, and Hsp20), Hsp20 or small Hsps (sHsps) are expressed in maximal amounts under high temperature stress. The characteristic feature of the sHsps is the presence of alpha-crystallin domain (ACD) at the C-terminus. sHsps cooperate with Hsp100/Hsp70 and cochaperones in ATP-dependent manner in preventing aggregation of cellular proteins and in their subsequent refolding. A database search revealed the presence of 40 alpha-crystallin domain containing genes in rice. Phylogenetic analysis showed that 23 out of these 40 genes constitute sHsps. The additional 17 genes containing ACD clustered with Acd proteins of *Arabidopsis*. A detailed scrutiny of 23 sHsp sequences resulted in categorizing these proteins in a revised scheme of classification, constituting 16 cytoplasmic/nuclear, 2 ER, 3 mitochondrial, 1 plastid, and 1 peroxisomal genes. Expression analysis based on microarray and RT-PCR showed that 19 sHsp genes were upregulated by high-temperature stress. Besides heat stress, expression of sHsp genes was up- or downregulated by other abiotic and biotic stresses [14].

Several studies have pointed out the involvement of WRKY transcription factor gene family in a range of biological processes, including abiotic stress. A whole gene family WRKYs expression study was carried out in rice. Twenty-four members of the rice WRKY gene family (22% of the total) were differentially regulated in response to at least one of the stress conditions tested. The existence of nine OsWRKY gene clusters comprising phylogenetically related and unrelated genes that were significantly coexpressed suggested that specific sets of WRKY genes might act in coregulatory networks. By identifying *Arabidopsis* orthologues of the coexpressed OsWRKY genes, it was also shown that specific coregulatory networks were conserved between the two model species. It is possible to highlight novel clusters of plant genes contributing to the same biological processes or signal transduction pathways using such data [15].

Similar studies have also been conducted in wheat, although limited by the unavailability of the whole-genome sequence. From 960 174 ESTs of *T. aestivum*, 117 putative AP2/ERF family genes were identified [16]. On the basis of the model species *A. thaliana*, the AP2/ERF transcription factors from *T. aestivum* were classified into five subfamilies with the following number of members: DREB (57), ERF (47), AP2 (9), RAV (3), and Soloist (1). Using the available EST information as a source of expression data, the putative AP2/ERF family genes from *T. aestivum* were detected in nine kinds of tissues. Transcripts of the genes were shown to be most abundant in leaves, followed by roots and seeds, and least abundant in stem.

Enzymatic methylation, which is catalyzed by the large number of *O*-methyltransferases (OMTs), is one of the important reactions in the flow of primary and secondary metabolism. The structural and expressional divergence of genes encoding *O*-methyltransferase has been studied in wheat [17]. Wheat OMT genes TaOMT3, TaOMT4, and TaOMT5 were analyzed using a bioinformatics approach for their genomic organization, tissue-specific expression, responses to abiotic stresses and hormones, and *cis*-elements.

30.3 Transcriptomics

With the ever-increasing availability of genomic sequences and the introduction of microarray technology, enabling the high-throughput analysis of gene expression, transcriptome profiling has rapidly become a favorite tool with many researchers. The study of plant transcriptomes has led to important discoveries and to an accumulation of profiling data covering a wide range of different tissues, developmental stages, perturbations, and genotypes. Querying a large number of microarray experiments can provide insights that cannot be gained by analyzing single experiments.

In an effort to elucidate genome-level responses to drought and high-salinity stress in rice, a 70-mer oligomer microarray covering 36 926 unique genes or gene models was used to profile genome expression changes in rice shoot, flag leaf, and panicle under drought or high-salinity conditions [18]. Patterns of gene expression

in response to drought or high-salinity stress within a particular organ type showed significant overlap, but comparison of expression profiles among different organs showed largely organ-specific patterns of regulation. Both stresses appeared to alter the expression patterns of a significant number of genes involved in transcription and cell signaling in a largely organ-specific manner. This study identified that promoter regions of genes induced by both stresses or induced by one stress in more than one organ type were relatively enriched in two *cis*-elements (ABRE core and DRE core) known to be associated with water stress. Further computational analysis that indicated that novel promoter motifs are present in the promoters of genes involved in rehydration after drought led the authors to propose that rice might possess a mechanism that actively detects rehydration and facilitates rapid recovery.

The rice genome encodes a total of 10 genes that contain the highly conserved MTase catalytic domains found in DNA methyltransferases (MTases). A microarray-based gene expression profile of all 10 MTases during 22 stages/tissues that included 14 stages of reproductive development and five vegetative tissues together with three stresses, cold, salt, and dehydration stress, revealed specific windows of MTase activity during panicle and seed development. One of the MTases was activated in young seedlings in response to cold and salt stress [19].

Microarray-based transcriptome profiling has been successfully used to analyze the differences in gene expression between salt-sensitive and -tolerant rice cultivars. The expression profiles of 1194 salinity-regulated cDNAs in rice salt-sensitive cultivar IR64 and Pokkali, a well-known, naturally salt-tolerant relative, were analyzed using microarrays [20]. The study revealed that salinity tolerance of Pokkali may be due to constitutive overexpression of many genes that function in salinity tolerance. Analysis of genome architecture revealed the presence of these genes on all the chromosomes with several distinct clusters. A few genes were mapped on one of the major quantitative trait loci, Saltol, on chromosome 1 and were found to be differentially regulated in the two contrasting genotypes. The study revealed that a set of known abiotic stress-inducible genes, including CaMBP, GST, LEA, V-ATPase, OSAP1 zinc finger protein, and transcription factor HBP1B, were expressed at high levels in Pokkali even in the absence of stress.

Mitochondrial responses to abiotic stresses at the early stages of wheat development after imbibition under normal and low temperature, high salinity, and high osmotic potential stress have been evaluated by transcriptome profiling [21]. Microarray analysis of the mitochondrial transcriptome revealed stress specific in transcript levels in the case of most genes, but few groups of genes were found to respond commonly to different stresses. Under continuous stresses for 3 days, 13 genes showed low-temperature-specific responses with up- or downregulation, while 14 and 23 genes showed responses specific to high salinity and high osmotic potential, respectively. On the other hand, 13 genes showed common responses. Among the nuclear-encoded mitochondrial-targeted genes, MnSOD and AOX increased their transcript amounts. These results also point out toward common and different regulatory mechanisms that can sense different abiotic stresses and modulate both nuclear and mitochondrial gene expression in wheat.

In order to reveal differences in global expression profiles of drought-tolerant and -sensitive wild emmer wheat genotypes, a shock-like dehydration process was deployed to compare transcriptomes at two time points in root and leaf tissues [22]. The comparison of transcriptomes revealed several unique genes or expression patterns such as differential usage of IP_3-dependent signal transduction pathways, ethylene- and abscisic acid (ABA)-dependent signaling, and preferential or faster induction of ABA-dependent transcription factors by the tolerant genotype that distinguish contrasting genotypes indicative of distinctive stress response pathways. This comparison of transcriptomes in the absence of and after dehydration indicates that gene networks involved in drought response especially in root tissues may have been lost in the selection processes generating modern bread wheat.

T. aestivum "Opata" is an elite hard red spring wheat that has been used as a parent of the ITMI (International *Triticeae* Mapping Initiative) mapping population and also in the production of synthetically derived hexaploid wheats, some of which (following selection) show increased drought tolerance relative to Opata. The response of Opata roots to water withholding was described using physiological variables and oligonucleotide microarrays [23]. They identified 190 transcripts whose expression increased following water limitation. In addition to previously characterized markers of abiotic stress and many genes of unknown function, they were able to identify multiple putative glucanases and class III peroxidases as being particularly responsive to stress. A comparison of these data with microarray analyses of Opata's more drought-tolerant, synthetic-derived Progeny revealed a relatively high correlation between responsive transcripts in the two genotypes, despite differing physiological responses. Some of the transcripts that were differentially expressed between Opata and the more tolerant synthetic-derived genotype under stress included a class III peroxidase, an AP2 family transcription factor, and several transcripts of unknown function.

There are a few software applications that have been developed to query large microarray gene expression databases using a Web-browser interface. GENEVESTIGATOR, a database and Web-browser data mining interface for Affymetrix GeneChip data, is an example. Users can query the database to retrieve the expression patterns of individual genes throughout chosen environmental conditions, growth stages, or organs. Reversely, mining tools allow users to identify genes specifically expressed during selected stresses, growth stages, or in particular organs. Using GENEVESTIGATOR, the gene expression profiles of more than 22 000 *Arabidopsis* genes can be obtained, including those of 10 600 currently uncharacterized genes [24]. More recently, Genevestigator rice and barley gene expression databases have been released that contain quality controlled and well-annotated microarray experiments using ontologies [25]. The databases comprise experiments from pathology, plant nutrition, abiotic stress, hormone treatment, genotype, and spatial or temporal analysis, but are expected to cover a broad range of research areas as more experimental data become available. The transcriptome meta-analysis of the model species rice is expected to deliver results that can be used for functional genomics and biotechnological applications in cereals.

In addition to the whole transcriptome profiling studies, a plethora of research reports are recently available on transcript profiling of individual genes or specific

class of genes functioning in abiotic stress tolerance in wheat and rice. These studies complement the whole transcriptome profiling studies and in many cases are more in-depth when individual gene's function is concerned. Many of them have served to reveal novel functions for already known genes and help in elucidating their interactions with other genes and pathways.

A recent review analyzed the role of APETALA 2/ethylene response element binding protein (AP2/EREBP) family transcription factors in abiotic stress tolerance. *O. sativa* subsp. *Japonica* has 163 gene loci assigned to this transcription factor. AP2/EREBP transcription factors have been implicated in hormone, sugar, and redox signaling in context of abiotic stresses such as cold and drought. It was suggested that AP2/EREBP transcription factors integrate metabolic, hormonal, and environmental signals in stress acclimation and retrograde signaling [26].

Many transcription factors involved in abiotic stress tolerance in an ABA-dependant manner have been characterized. Two group A bZIP transcription factors in rice, OsABF1 and OsABF2 (*O. sativa* ABA-responsive element binding factor), were found to be expressed in various tissues in rice and induced by different types of abiotic stress treatments, such as drought, salinity, cold, oxidative stress, and ABA [27, 28]. MYBS3 is a single DNA binding repeat MYB transcription factor previously shown to mediate sugar signaling in rice. A recent study revealed that MYBS3 also plays a critical role in cold adaptation in rice. Transgenic rice constitutively overexpressing MYBS3 tolerated 4 °C for at least 1 week and exhibited no yield penalty in normal field conditions. Transcription profiling of transgenic rice overexpressing or underexpressing MYBS3 led to the identification of many genes in the MYBS3-mediated cold signaling pathway. MYBS3 was found to repress the well-known DREB1/CBF-dependent cold signaling pathway in rice, and the analysis revealed that the repression appears to act at the transcriptional level. DREB1 responded quickly and transiently while MYBS3 responded slowly to cold stress, which suggests that distinct pathways act sequentially and complementarily for adapting short- and long-term cold stress in rice [29].

Differences in expression pattern of two abiotic stress-inducible genes in contrasting rice genotypes varying in their salt stress sensitivity were studied [30]. Expression levels of two genes, Rab16A and SamDC, and corresponding proteins, in the seeds, at the background level (dry or water-imbibed state) and ABA-imbibed conditions in rice genotypes M-1-48 (salt sensitive), Nonabokra (salt tolerant), and Gobindobhog (aromatic) were analyzed. An extremely low abundance of Rab16A or practically undetectable SamDC transcripts were observed in M-1-48 and Gobindobhog seeds under control conditions, induced only after exogenous ABA treatment, whereas they were expressed at a much higher level even in dry and water-imbibed seeds of Nonabokra and lesser induced by ABA. The RAB16A and SAMDC protein expression in the three varieties were also identical to the gene expression patterns. Thus, the expression was found to be stress inducible in M-1-48 and Gobindobhog, while constitutive in Nonabokra. Their results indicated that the difference in expression profiles of the two genes is partly responsible for increased salt tolerance in Nonabokra.

In an effort to characterize previously uncharacterized rice genes, a large population of *Arabidopsis* plants were transformed with rice full-length cDNAs to isolate the rice genes that improve the tolerance of plants to environmental stress [31]. One salt-tolerant line identified, R07047, expressed a rice gene, OsSMCP1, which encodes a small protein with a single C2 domain, a Ca^{2+}-dependent membrane targeting domain. Line R07047 showed enhanced tolerance to high salinity, osmotic, dehydrative, and oxidative stresses. Furthermore, R07047 showed improved resistance to *P. syringae*. *In vivo* localization studies revealed the plastid localization of the protein. Overexpression of OsSMCP1 was found to induce overexpression of several nuclear-encoded genes, including the stress-associated genes, in transgenic *Arabidopsis* [31].

In similar experiments, the role of a previously unknown zinc finger transcription factor called DST (drought and salt tolerance) that negatively regulates stomatal closure by direct modulation of genes related to H_2O_2 homeostasis in drought and salt tolerance was analyzed [32]. A DREB1B gene (dehydration-responsive element binding factor) from rice, differentially regulated at the transcriptional level by osmotic stress, oxidative stress, salicylic acid, ABA, and cold, was characterized by overexpression in tobacco plants and analysis of transgenic plants [33]. Another study revealed the involvement of specific MAP kinase kinase (mitogen-activated protein kinase) in different abiotic stress signaling [34]. Two rice calmodulin promoters OsCaM1 and OsCaM3 were characterized by fusion with GUS reporter gene and transformation into tobacco [35].

A study in two wheat recombinant inbred lines contrasting in their salt tolerance examined the expression levels under salt or drought stress of 10 MYB transcription factor genes [36]. Four MYB genes were found to be consistently upregulated in the seedling roots of both genotypes under short-term salt treatment. Three MYB genes were found to be upregulated in both genotypes under long-term salt stress. One MYB gene was upregulated in both genotypes under both short- and long-term salt stress. Of these salt upregulated MYB genes, one MYB gene (TaMYBsdu1) was markedly upregulated in the leaf and root of wheat under long-term drought stress. In addition, TaMYBsdu1 showed higher expression levels in the salt-tolerant genotype than in the susceptible genotype under salt stress, indicating that it is an important regulator involved in wheat adaptation to both salt and drought stresses.

A novel wheat NAC transcription factor gene (TaNAC4) was found to share high homology with rice OsNAC4 gene. TaNAC4 transcript in wheat leaves was induced by the infection of strip rust pathogen and also by exogenously applied methyl jasmonate (MeJA), ABA, and ethylene. Environmental stimuli, including high salinity, wounding, and low temperature, also induced TaNAC4 expression [37]. A pectin methylesterase inhibitor (PMEI) was found to be upregulated under hydrogen peroxide treatments in wheat [38]. Dehydration-responsive element binding factors (DBFs) belong to the AP2/ERF superfamily and play vital regulatory roles in abiotic stress responses in plants. Three novel homologues of the DBF gene family in wheat (TaAIDa-c, *T. aestivum* abiotic stress-induced DBFs) were isolated by screening a drought-induced cDNA library [39]. Overexpression of TaAIDFa activated CRT/DRE-containing genes under normal growth conditions and improved drought

and osmotic stress tolerances in transgenic *Arabidopsis* plants. Vacuolar H^+-translocating pyrophosphatase (V-PPase) is a key enzyme related to both plant growth and abiotic stress tolerance. V-PPase genes TaVP1, TaVP2, and TaVP3 were identified from wheat [40]. TaVP2 was observed to be mainly expressed in shoot tissues and downregulated in leaves under dehydration. Its expression was upregulated in roots under high salinity. TaVP1 was relatively more ubiquitously and evenly expressed than TaVP2. Its expression level in roots was highest among the tissues examined and was inducible by salinity stress. These results indicated that the V-PPase gene paralogues in wheat are differentially regulated spatially and in response to dehydration and salinity stresses.

Sucrose nonfermenting 1-related protein kinase 2 family members play essential roles in response to hyperosmotic stresses in *Arabidopsis*, rice, and maize. A study characterized the function of TaSnRK2.4, an SNF1-type serine/threonine protein kinase of wheat, in drought, salt, and freezing stresses in *Arabidopsis* [41]. Transgenic *Arabidopsis* overexpressing TaSnRK2.4 had enhanced tolerance to drought, salt, and freezing stresses, which were simultaneously supported by physiological results, including decreased rate of water loss, enhanced higher relative water content, strengthened cell membrane stability, improved photosynthesis potential, and significantly increased osmotic potential. The results indicated that TaSnRK2.4 is involved in the regulation of enhanced osmotic potential, growth, and development under both normal and stress conditions and imply that TaSnRK2.4 is a multifunctional regulatory factor in *Arabidopsis*.

A novel aquaporin gene from wheat, TaNIP (*T. asetivum* L. nodulin 26-like intrinsic protein), was characterized in a study [42]. TaNIP was identified and cloned through the GeneChip expression analysis of a salt-tolerant wheat mutant RH8706-49 under salt stress. The overexpression of TaNIP in transgenic *Arabidopsis* produced higher salt tolerance than wild-type plants. Under salt stress treatment, TaNIP overexpressing *Arabidopsis* accumulated higher K^+, Ca^{2+}, and proline contents and lower Na^+ level than the wild-type plants. The overexpression of TaNIP in transgenic *Arabidopsis* also upregulated the expression of a number of stress-associated genes. Their results suggested that TaNIP plays an important role in salt tolerance.

30.4 Evaluation of the Role of MicroRNAs in Abiotic Stress

MicroRNAs (miRNAs) are small single-stranded RNAs with a length of about 21 nt; these noncoding RNAs regulate developmental and stress responses in plants by cleaving mRNAs. Most of the physiological processes are controlled by miRNAs in several organisms including plants. A huge database exists on miRNAs identified from diverse species. However, the processes of data mining of miRs in most of the species are still incomplete. Although many miRNAs have been identified in rice and wheat, relatively little is known about their role in abiotic stress.

Cloning and identification of approximately 40 new putative miRs is reported from a basmati rice variety [43]. About 23 sequences were derived from rice exposed to salt

stress, while 18 were derived from rice infected with tungro virus. A few of these putative miRs were common to both. Their results showed that at least two of these miRs were upregulated in response to both abiotic and biotic stresses. The miR target predictions indicated that most of the putative miRs target specific metabolic processes. The upregulation of similar miRs in response to two entirely different types of stresses suggests a converging functional role of miRs in managing various stresses. Recently, 18 cold-responsive rice miRNAs were identified using microarrays [44]. The existence of hormone-responsive elements in the upstream regions of these cold-responsive miRNAs indicated the importance of hormones in this defense system mediated by miRNAs. Their findings confirm the role of miRNAs as ubiquitous regulators in rice.

Deep sequencing of small RNA libraries is an effective approach to uncover rare and lineage- and species-specific miRNAs in any organism. A study constructed three small RNA libraries from control rice seedlings and seedlings exposed to drought or salt stress and then subjected them to pyrosequencing [45]. A total of 58 781, 43 003, and 80 990 unique genome-matching small RNAs were obtained from the control, drought, and salt stress libraries, respectively. Twenty-three new miRNAs, mostly each derived from a unique locus in rice genome, were identified. Six of the new miRNAs are conserved in other monocots.

In wheat, 12 miRNAs responsive to heat stress have been identified [46]. Another study identified 2076 small RNAs in a small RNA library from leaf, root, and spike of wheat. These small RNAs mapped to noncoding regions, the CDS region of protein-coding genes, and 5′ UTR and 3′ UTR regions. The expression of small RNAs in wheat seedling leaves, roots, and spikes were analyzed by Northern blot, which indicated that some small RNAs were responsive to abiotic stress treatments, including heat, cold, salt, and dehydration [47].

30.5
Generation of Transgenic Wheat and Rice Plants Tolerant to Abiotic Stress

Transgenic rice plants overexpressing ZFP245, a cold- and drought-responsive gene that encodes a zinc finger protein, were found to display high tolerance to cold and drought stresses. The transgenic plants did not exhibit growth retardation, but showed growth sensitivity against exogenous abscisic acid, increased free proline levels, and elevated expression of rice pyrroline-5-carboxylate synthetase and praline transporter genes under stress conditions. Overproduction of ZFP245 enhanced the activities of reactive oxygen species scavenging enzymes under stress conditions and increased the tolerance of rice seedlings to oxidative stress. It was postulated that ZFP245 may contribute to the tolerance of rice plants to cold and drought stresses by regulating proline levels and reactive oxygen species scavenging activities [48].

OsbZIP72, a member of the basic leucine zipper (bZIP) transcription factor family, is an ABRE binding factor in rice. Transgenic rice overexpressing OsbZIP72 showed a hypersensitivity to ABA, elevated levels of expression of ABA response

gene such as LEAs, and an enhanced ability of drought tolerance. These results suggested that OsbZIP72 plays a positive role in drought resistance through ABA signaling [49]. Transgenic rice overexpressing OsbZIP23, another member of the bZIP family, showed significantly improved tolerance to drought and high-salinity stresses and sensitivity to ABA. GeneChip and real-time polymerase chain reaction analyses revealed that hundreds of genes were up- or downregulated in the rice plants overexpressing OsbZIP23, indicating that OsbZIP23 is a major player of the bZIP family in rice for conferring ABA-dependent drought and salinity tolerance [50].

The DREB transcription factors, which specifically interact with C-repeat/DRE (A/GCCGAC), play an important role in plant abiotic stress tolerance by controlling the expression of many cold and drought-inducible genes in an ABA-independent pathway. Three novel DREB genes, OsDREB1E, OsDREB1G, and OsDREB2B, were isolated from rice [51]. Transgenic rice plants analysis revealed that overexpression of OsDREB1G and OsDREB2B in rice significantly improved their tolerance to water deficit stress, while overexpression of OsDREB1E could only slightly improve the tolerance to water deficit stress, suggesting that the OsDREBs might participate in the stress response pathway in different manners.

Overexpression of TERF1 (encoding a tomato ethylene response factor) in transgenic rice resulted in an increased tolerance to drought and high salt [52]. The authors associated the enhanced tolerance with the accumulation of proline and the decrease in water loss. Furthermore, TERF1 was found to effectively regulate the expression of stress-related functional genes Lip5, Wcor413-l, OsPrx, and OsABA2, as well as regulatory genes OsCDPK7, OsCDPK13, and OsCDPK19, under normal growth conditions. *cis*-Acting elements such as DRE/CRT and GCC box exist in TERF1 targeted gene promoters. Similarly, overexpression of a trehalose-6-phosphate phosphatase gene OsTPP1 conferred stress tolerance in rice and resulted in the activation of stress-responsive genes [53].

An aluminum (Al^{3+}) tolerance gene TaALMT1 was overexpressed in transgenic wheat under maize ubiquitin promoter [54]. The transgenics showed increased TaALMT1 expression, malate efflux, and Al^{3+} resistance compared to untransformed controls. Some T_2 lines showed greater Al^{3+} resistance than ET8, an Al^{3+}-resistant reference genotype. Increased drought tolerance was reported in transgenic wheat expressing *Vigna aconitifolia* Δ^1-pyrroline-5-carboxylate synthetase (P5CS) cDNA that encodes the key regulatory enzyme in proline biosynthesis under the control of a stress-induced promoter complex (AIPC) [55]. Drought stress tolerance was accompanied by accumulation of proline in transgenic plants.

30.6 Proteomics

The transcriptome analyses of gene expression at the mRNA level have contributed greatly to our understanding of abiotic stress tolerance in plants. However, the level of mRNA does not always correlate well with the level of protein, the key player in the

cell [56]. Therefore, it is insufficient to predict protein expression level from quantitative mRNA data. This is mainly due to posttranscriptional regulation mechanisms, such as nuclear export and mRNA localization, transcript stability, translational regulation, and protein degradation. Proteome studies aim at the complete set of proteins encoded by the genome and thus complement the transcriptome studies.

Several researchers have used the proteomics approach to identify specific proteins involved in rice stress response. The proteome response of the plasma membrane (PM) to environmental stresses was studied using a subcellular proteomics approach, monitoring changes in abundance of PM-associated protein in response to salinity [57]. The proteome was extracted from a root plasma membrane-rich fraction of a rice salt-tolerant variety, IR651, grown under saline and normal conditions. Comparative two-dimensional electrophoresis revealed that 24 proteins were differentially expressed in response to salt stress. Most of the proteins identified were involved in several important mechanisms of plant adaptation to salt stress, including regulation of PM pumps and channels, membrane structure, oxidative stress defense, signal transduction, protein folding, and the methyl cycle. These results point to the suitability of proteomics approach in identification of stress-regulated proteins.

In another study, a proteomic approach was adopted to investigate the low-abundant proteins in rice leaf in response to cold stress. Rice seedlings were exposed to different temperatures, such as 5 or 10 °C, and samples were collected after different time courses. The researchers were able to identify some novel proteins, such as cysteine proteinase, thioredoxin peroxidase, a RING zinc finger protein-like, drought-inducible late-embryogenesis abundant, and a fibrillin-like protein [58]. Another group of researchers also used proteomics approaches to get new insights into chilling stress responses in rice. Rice cultivar Nipponbare was treated at 6 °C for 6 or 24 h and then allowed to recover for 24 h. The temporal changes in total proteins in rice leaves were examined using two-dimensional electrophoresis [59]. The researchers were able to identify 85 differentially expressed proteins using mass spectrometry analysis that were involved in several processes including signal transduction, RNA processing, translation, protein processing, redox homeostasis, photosynthesis, photorespiration, and metabolisms of carbon, nitrogen, sulfur, and energy. Interestingly, gene expression analysis of 44 different proteins by quantitative real-time PCR showed that the mRNA level was not correlated well with the protein level. This underlines the importance of proteomics in identification of key components in stress tolerance. The same group had previously used proteomic successfully to investigate the salt stress-responsive proteins in rice cv. Nipponbare roots. They were able to identify six novel stress-responsive proteins involved in regulation of carbohydrate, nitrogen, and energy metabolism, reactive oxygen species scavenging, mRNA and protein processing, and cytoskeleton stability [56].

Several studies have suggested a critical role of protein phosphorylation in salt stress response in plants. A study analyzed the differential expression of rice phosphoproteome under salt stress [60]. Seventeen differentially upregulated and eleven differentially downregulated putative phosphoproteins were identified. The same group further identified 31 salt stress differentially regulated proteins, the

majority of which have not been reported in the literature. Thus, proteomics is indeed a valuable tool in providing new insight into plant response to abiotic stress.

Cell wall proteins (CWPs) are important both for maintenance of cell structure and for responses to abiotic and biotic stresses. To determine differentially expressed CWPs in wheat under flooding stress, gel-based proteomic and LC–MS/MS-based proteomic techniques were used [61]. Eighteen proteins were found to be significantly regulated in response to flood by gel-based proteomics and 15 proteins by LC–MS/MS-based proteomics. Among the flooding downregulated proteins, most were related to the glycolysis pathway and cell wall structure and modification. However, the most highly upregulated proteins in response to flooding belong to the category of defense and disease response proteins. Among these differentially expressed proteins, only methionine synthase, beta-1,3-glucanases, and beta-glucosidase were consistently identified by both techniques. The downregulation of these three proteins suggested that wheat seedlings respond to flooding stress by restricting cell growth to avoid energy consumption; by coordinating methionine assimilation and cell wall hydrolysis, CWPs played critical roles in flooding responsiveness. The implication of different drought treatments on the protein fractions in grains of winter wheat was examined using ^{1}H nuclear magnetic resonance spectroscopy followed by chemometric analysis [62]. Principal component trajectories of the total protein content and the protein fractions of flour, as well as the ^{1}H NMR spectra of single wheat kernels, wheat flour, and wheat methanol extracts, were analyzed to elucidate the metabolic development during grain filling. The results from both the ^{1}H NMR spectra of methanol extracts and the ^{1}H HR-MAS NMR of single kernels showed that a single drought event during the generative stage had as strong an influence on protein metabolism as two consecutive events of drought. In contrast, a drought event at the vegetative growth stage had little effect on the parameters investigated.

30.7 Metabolomics

Even after the completion of the whole-genome sequencing in many plants, networks of gene to metabolite are largely unknown. To reveal the function of genes involved in metabolic processes and gene-to-metabolite networks, the metabolomics-based approach is regarded as a direct way. In particular, integration of comprehensive gene expression profile with targeted metabolite analysis is shown to be an innovative way for identification of gene function for specific product accumulation in plants [63].

Metabolomics represents the exhaustive profiling of metabolites contained in organism. Proteomics and transcriptomics are both considered to be a flow of media concerning genetic information. In contrast, metabolomic should be thought as being concerned with phenotype [64]. Perturbations including environmental change, physical stress, abiotic stress, nutritional stress, mutation, and so on lead

to changes in the metabolome. Analysis of these changes serves to fine-tune our knowledge on plant response to environmental changes, physical stress, abiotic stress, nutritional stress, mutation, and so on.

Capillary electrophoresis–mass spectrometry (CE–MS) and capillary electrophoresis diode–array detection (CE–DAD) were used to analyze the dynamic changes in the level of 56 basic metabolites in rice foliage at hourly intervals over a 24 h period [65]. They found that in response to environmental stress, glutathione and spermidine fluctuated synchronously with their regulatory targets.

Overexpressing YK1gene, the homologue of the HC toxin reductase (HCTR) gene, in transgenic rice was accompanied by an increase in the amounts of NAD(P)(H). Besides HCTR activity, YK1 also possessed dihydroflavonol-4-reductase activity [66]. The overexpression of YK1 was found to induce the activation of enzymes in the NAD synthetic pathway, which resulted in an increase in the amount of NAD(P)(H). These results implied that the coupled increase in DFR activity and amounts of NAD(P)(H) may contribute to biotic and abiotic stress tolerance. A metabolite profiling of YK1 transgenic rice was done by CE/MS [67]. They analyzed several metabolites of glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway. In addition, the concentrations of sugars and ions were quantified. Their results indicated that in YK1 overexpressing plants, the concentrations of *cis*-aconitate, isocitrate, and 2-oxoglutarate were higher in leaves, whereas those of fructose-1,6-bisphosphate and glyceraldehyde-3-phosphate were lower in roots. In seeds, the amounts of free amino acids and metals were altered, whereas sugars in seeds were kept constant. While the overexpression of YK1 was associated with only slight changes in the amounts of several metabolites analyzed, glutathione derivatives were substantially increased in suspension cultured cells.

Impact of nitrogen (N) and sulfur (S) deficiency on N and S remobilization from senescing canopy tissues during grain filling in winter wheat was studied using transcriptomic and metabolomic approaches [68]. Nuclear magnetic resonance (NMR) metabolite profiling revealed significant effects of suboptimal N or S supply in leaves but not in developing grain. Analysis of amino acid pools in the grain and leaves revealed a strategy whereby amino acid biosynthesis switches to the production of glutamine during grain filling. Glutamine was found to accumulate in the first 7 days of grain development, prior to conversion to other amino acids and protein in the subsequent 21 days. Transcriptome analysis indicated downregulation of the terminal steps in many amino acid biosynthetic pathways. Their results indicated that vegetative tissue N has a greater control over the timing and extent of nutrient remobilization than S.

30.8
Conclusions and Perspectives

The progress of "omic" technologies during the past decade has been spectacular. After sequencing the complete genome of *Arabidopsis* in 2002, the technology has

catapulted, significantly reducing the time and cost required to sequence the complete genome of a higher plant. The next-generation sequencing technologies have generated an information explosion that scientists all over the world are earnestly exploring. Genomics, transcriptomics, proteomics, and metabolomics investigate different facets of a given scientific issue such as abiotic stress tolerance, but complement each other. Integration of phenotypic, genetic, transcriptomic, proteomic, and metabolomic data will enable accurate and detailed gene network reconstruction. This will ultimately result in the elucidation of the molecular pathways involved in complex phenotypic traits. A better understanding of genetic and cellular mechanisms behind abiotic stress tolerance would facilitate generation of transgenic plants with desired traits with little or no undesired/unforeseen effects.

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References

1 Bray, E.A., Bailey-Serres, J., and Weretilnyk, E. (2000) Responses to abiotic stresses, in *Biochemistry and Molecular Biology of Plants* (eds W. Gruissem, B. Buchannan, and R. Jones), American Society of Plant Physiologists, Rockville, pp. 1158–1249.

2 George, S. and Parida, A. (2008) *Nutr. Nat. Resour.*, **3**, 13–20.

3 Gill, B.S., Appels, R., Botha-Oberholster, A.M., Buell, C.R., Bennetzen, J.L., Chalhoub, B., Chumley, F., Dvorák, J., Iwanaga, M., Keller, B., Li, W., McCombie, W.R., Ogihara, Y., Quetier, F., and Sasaki, T.A. (2004) *Genetics*, **168**, 1087–1096.

4 Yamaguchi, T. and Blumwald, E. (2005) *Trends Plant Sci.*, **10**, 1360–1385.

5 Bennett, M.D. and Leitch, I.J. (1995) *Ann. Bot.*, **73**, 113–176.

6 Sasaki, T. (2003) *Breed. Sci.*, **53**, 281–289.

7 Kawaura, K., Mochida, K., Enju, A., Totoki, Y., Toyoda, A., Sakaki, Y., Kai, C., Kawai, J., Hayashizaki, Y., Seki, M., Shinozaki, K., and Ogihara, Y. (2009) *BMC Genomics*, **10**, 271.

8 Houde, M., Belcaid, M., Ouellet, F., Danyluk, J., Monroy, A.F., Dryanova, A., Gulick, P., Bergeron, A., Laroche, A., Links, M.G., MacCarthy, L., Crosby, W.L., and Sarhan, F. (2006) *BMC Genomics*, **7**, 149.

9 Sorrells, M.E., La Rota, M., Bermudez-Kandianis, C.E., Greene, R.A., Kantety, R., Munkvold, J.D., *et al.* (2003) *Genome Res.*, **13**, 1818–1827.

10 Jain, M., Ghanashyam, C., and Bhattacharjee, A. (2010) *BMC Genomics*, **11**, 73.

11 Ramamoorthy, R., Jiang, S.Y., Kumar, N., Venkatesh, P.N., and Ramachandran, S. (2008) *Plant Cell Physiol.*, **49**, 865–879.

12 Matsukura, S., Mizoi, J., Yoshida, T., Todaka, D., Ito, Y., Maruyama, K., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2010) *Mol. Genet. Genomics*, **283**, 185–196.

13 Ma, K., Xiao, J., Li, X., Zhang, Q., and Lian, X. (2009) *Gene*, **444**, 33–45.

14 Sarkar, N.K., Kim, Y.K., and Grover, A. (2009) *BMC Genomics*, **10**, 393.

15 Berri, S., Abbruscato, P., Faivre-Rampant, O., Brasileiro, A.C., Fumasoni, I., Satoh, K., Kikuchi, S., Mizzi, L., Morandini, P., Pè, M.E., and

Piffanelli, P. (2009) *BMC Plant Biol.*, **9**, 120.
16 Zhuang, J., Chen, J.M., Yao, Q.H., Xiong, F., Sun, C.C., Zhou, X.R., Zhang, J., and Xiong, A.S. (2010) *Mol. Biol. Rep.*, **38**, 745
17 Jung, J.H., Hong, M.J., Kim, D.Y., Kim, J.Y., Heo, H.Y., Kim, T.H., Jang, C.S., and Seo, Y.W. (2008) *Genome*, **51**, 856–869.
18 Zhou, J., Xiangfeng, W., Yuling, J., Yonghua, Q., Xigang, L., Kun, He., *et al.* (2007) *Plant Mol. Biol.*, **63**, 591–608.
19 Sharma, R., Mohan Singh, R.K., Malik, G., Deveshwar, P., Tyagi, A.K., Kapoor, S., and Kapoor, M. (2009) *FEBS J.*, **276**, 6301–6311.
20 Kumari, S., Sabharwal, V.P., Kushwaha, H.R., Sopory, S.K., Singla-Pareek, S.L., and Pareek, A. (2009) *Funct. Integr. Genomics*, **9**, 109–123.
21 Naydenov, N.G., Khanam, S., Siniauskaya, M., and Nakamura, C. (2010) *Genes Genet. Syst.*, **85**, 31–42.
22 Ergen, N.Z., Thimmapuram, J., Bohnert, H.J., and Budak, H. (2009) *Funct. Integr. Genomics*, **9**, 377–396.
23 Mohammadi, M., Kav, N.N., and Deyholos, M.K. (2008) *Genome*, **51**, 357–367.
24 Grennan, A.K. (2006) *Plant Physiol.*, **140**, 1139–1141.
25 Zimmermann, P., Laule, O., Schmitz, J., Hruz, T., Bleuler, S., and Gruissem, W. (2008) *Mol. Plant*, **1**, 851–857.
26 Dietz, K.J., Vogel, M.O., and Viehhauser, A. (2010) *Protoplasma*, **245**, 3–14
27 Hossain, A.M., Lee, Y., Cho, J.I., Ahn, C.H., Lee, S.K., Jeon, J.S., Kang, H., Lee, C.H., An, G., and Park, P.B. (2010) *Plant Mol. Biol.*, **2**, 557–566.
28 Hossain, M.A., Cho, J.I., Han, M., Ahn, C.H., Jeon, J.S., An, G., and Park, P.B. (2010) *J. Plant Physiol.*, **167**, 1512–1520.
29 Su, C.F., Wang, Y.C., Hsieh, T.H., Lu, C.A., Tseng, T.H., and Yu, S.M. (2010) *Plant Physiol.*, **153**, 145–158.
30 Roychoudhury, A., Basu, S., and Sengupta, D.N. (2009) *Indian J. Exp. Biol.*, **47**, 827–833.
31 Yokotani, N., Ichikawa, T., Kondou, Y., Maeda, S., Iwabuchi, M., Mori, M., Hirochika, H., Matsui, M., and Oda, K. (2009) *Plant Mol. Biol.*, **71**, 391–402.
32 Huang, X.Y., Chao, D.Y., Gao, J.P., Zhu, M.Z., Shi, M., and Lin, H.X. (2009) *Genes Dev.*, **23**, 1805–1817.
33 Gutha, L.R. and Reddy, A.R. (2008) *Plant Mol. Biol.*, **68**, 533–555.
34 Kumar, K., Rao, K.P., Sharma, P., and Sinha, A.K. (2008) *Plant Physiol. Biochem.*, **46**, 891–897.
35 Jung, K.Y., Kyung, C.E., In, L.S., Lim, C.O., and Ju, C.Y. (2010) *BMB Rep.*, **43**, 9–16.
36 Rahaie, M., Xue, G.P., Naghavi, M.R., Alizadeh, H., and Schenk, P.M. (2010) *Plant Cell Rep.*, **29**, 835–844.
37 Xia, N., Zhang, G., Liu, X.Y., Deng, L., Cai, G.L., Zhang, Y., Wang, X.J., Zhao, J., Huang, L.L., and Kang, Z.S. (2010) *Mol. Biol. Rep.*, **37**, 3703–3712.
38 Hong-Bo, S., Zong-Suo, L., and Ming-An, S. (2005) *Colloids Surf. B Biointerfaces*, **45**, 7–13.
39 Xu, Z.S., Ni, Z.Y., Liu, L., Nie, L.N., Li, L.C., Chen, M., and Ma, Y.Z. (2008) *Mol. Genet. Genomics*, **280**, 497–508.
40 Wang, Y., Xu, H., Zhang, G., Zhu, H., Zhang, L., Zhang, Z., Zhang, C., and Ma, Z. (2009) *J. Genet. Genomics*, **36**, 711–720.
41 Mao, X., Zhang, H., Tian, S., Chang, X., and Jing, R. (2010) *J. Exp. Bot.*, **61**, 683–696.
42 Gao, Z., He, X., Zhao, B., Zhou, C., Liang, Y., Ge, R., Shen, Y., and Huang, Z. (2010) *Plant Cell Physiol.*, **51**, 767–775.
43 Sanan-Mishra, N., Kumar, V., Sopory, S.K., and Mukherjee, S.K. (2009) *Mol. Genet. Genomics*, **282**, 463–474.
44 Lv, D.K., Bai, X., Li, Y., Ding, X.D., Ge, Y., Cai, H., Ji, W., Wu, N., and Zhu, Y.M. (2010) *Gene*, **459**, 39–47.
45 Sunkar, R., Zhou, X., Zheng, Y., Zhang, W., and Zhu, J.K. (2008) *BMC Plant Biol.*, **8**, 25.
46 Xin, M., Wang, Y., Yao, Y., Xie, C., Peng, H., Ni, Z., and Sun, Q. (2010) *BMC Plant Biol.*, **10**, 123.
47 Yao, Y., Ni, Z., Peng, H., Sun, F., Xin, M., Sunkar, R., Zhu, J.K., and Sun, Q. (2010) *Funct. Integr. Genomics*, **10**, 187–190.
48 Huang, J., Sun, S.J., Xu, D.Q., Yang, X., Bao, Y.M., Wang, Z.F., Tang, H.J., and

Zhang, H. (2009) *Biochem. Biophys. Res. Commun.*, **389**, 556–561.
49 Lu, G., Gao, C., Zheng, X., and Han, B. (2009) *Planta*, **229**, 605–615.
50 Xiang, Y., Tang, N., Du, H., Ye, H., and Xiong, L. (2008) *Plant Physiol.*, **148**, 1938–1952.
51 Chen, J.Q., Meng, X.P., Zhang, Y., Xia, M., and Wang, X.P. (2008) *Biotechnol. Lett.*, **30**, 2191–2198.
52 Gao, S., Zhang, H., Tian, Y., Li, F., Zhang, Z., Lu, X., Chen, X., and Huang, R. (2008) *Plant Cell Rep.*, **27**, 1787–1795.
53 Ge, L.F., Chao, D.Y., Shi, M., Zhu, M.Z., Gao, J.P., and Lin, H.X. (2008) *Planta*, **228**, 191–201.
54 Pereira, J.F., Zhou, G., Delhaize, E., Richardson, T., Zhou, M., and Ryan, P.R. (2010) *Ann. Bot.*, **106**, 205–214.
55 Vendruscolo, E.C., Schuster, I., Pileggi, M., Scapim, C.A., Molinari, H.B., Marur, C.J., and Vieira, L.G. (2007) *J. Plant Physiol.*, **164**, 1367–1376.
56 Yan, S., Tang, Z., Su, W., and Sun, W. (2005) *Proteomics*, **5**, 235–44.
57 Nohzadeh, M.S., Habibi, R.M., Heidari, M., and Salekdeh, G.H. (2007) *Biosci. Biotechnol. Biochem.*, **71**, 2144–2154.
58 Lee, D.G., Ahsan, N., Lee, S.H., Kang, K.Y., Lee, J.J., and Lee, B.H. (2007) *C. R. Biol.*, **330**, 215–225.
59 Yan, S.P., Zhang, Q.Y., Tang, Z.C., Su, W.A., and Sun, W.N. (2006) *Mol. Cell Proteomics*, **5**, 484–496.
60 Chitteti, B.R. and Peng, Z. (2007) *J. Proteome Res.*, **6**, 1718–1727.
61 Kong, F.J., Oyanagi, A., and Komatsu, S. (2010) *Biochim. Biophys. Acta*, **1804**, 124–136.
62 Winning, H., Viereck, N., Wollenweber, B., Larsen, F.H., Jacobsen, S., Søndergaard, I., and Engelsen, S.B. (2009) *J. Exp. Bot.*, **60**, 291–300.
63 Hirai, M.Y., Yano, M., Goodenowe, D., Kanaya, S., Kimura, T., Awazuhara, M., *et al.* (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 10205–10210.
64 Fukusaki, E. and Kobayashi, A. (2005) *J. Biosci. Bioeng.*, **100**, 347–354.
65 Sato, S., Soga, T., Nishioka, T., and Tomita, M. (2004) *Plant J.*, **40**, 151–163.
66 Hayashi, M., Takahashi, H., Tamura, K., Huang, J., Yu, L.H., Kawai-Yamada, M., *et al.* (2005) *Proc. Nat. Acad. Sci. USA*, **102**, 7020–7025.
67 Takahashi, H., Hotta, Y., Hayashi, M., Kawai-Yamada, M., Komatsu, S., and Uchimiya, H. (2005) *Plant Biotechnol.*, **22**, 47–50.
68 Howarth, J.R., Parmar, S., Jones, J., Shepherd, C.E., Corol, D.I., Galster, A.M., Hawkins, N.D., Miller, S.J., Baker, J.M., Verrier, P.J., Ward, J.L., Beale, M.H., Barraclough, P.B., and Hawkesford, M.J. (2008) *J. Exp. Bot.*, **59**, 3675–3689.

31
Rice: Genomics-Assisted Breeding for Drought Tolerance

Prashant Vikram, Arvind Kumar, Alok Singh, and Nagendra K. Singh

Rice is a major source of global food calories. Increasing population pressure, unpredictable rainfall patterns, shrinking fresh water resources, and increased frequency of severe drought spells in recent years are the reasons behind putting concerted efforts toward breeding drought-tolerant rice cultivars much needed by the rice farmers. Attempts to breed rice for stress environments have made limited progress so far, but success with recent research on identification of major QTL (quantitative trait loci) for grain yield under drought shows that genomics-assisted breeding could be a viable alternative to enhance grain yield under drought. Major QTL with consistent effects on grain yield under drought and different drought-related traits have been mapped on rice chromosome 1, 2, 3, 9, and 12 and search is on for the genes underlying these QTL. Direct selection for yield under drought has emerged as an important criterion for both conventional and molecular breeding approaches. Transgenic rice with overexpression of transcription factors such as *DREB1* and *SNAC1* has shown considerable promise, but its use in breeding is still impeded.

31.1
Introduction

Rice is the most important food crop of the world, cultivated in an area of about 150 million ha. Despite huge annual production of over 550 million ton, only about 4% of the total production is traded in international markets. In Asia, rice supplies 35–60% of the total food calories [1]. In countries where rice is the major food source, including China, India, and Indonesia, the average annual rate of population growth (1.7%) has been higher than 1.2% average growth in rice production [2]. According to FAO estimates, the world population would be around 9.8 billion by 2050 and 75% more food would be required to feed the additional population [3]. Global warming and unpredictable rainfall patterns in recent past have led to severe drought spells causing huge yield losses and severe shortage in food production in several parts of the world.

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Rice is a semiaquatic plant that is grown under four different cropping environments: irrigated, rain-fed lowland, rain-fed upland, and deep water [4]. Irrigated rice is by far the most common ecosystem constituting 55% of the acreage and 75% of the production globally [1]. The rain-fed lowland rice constitutes the second most important ecosystem sharing 25% of the global rice area. These are fields that do not receive irrigation water, but there are bunds around the fields that allow the water to accumulate on the field surface when there is rain [1]. Deep-water rice is planted in the areas that are naturally flooded during the rainy season. This rice represents about 8% of the total rice-growing area and here rice seeds are broadcasted a few weeks before the beginning of the monsoon season [1]. Rain-fed upland, also called dry land, rice is the type grown in areas where good soil drainage combined with an uneven land surface makes the accumulation of water impossible. Upland rice represents around 12% of world acreage and is the rice ecosystem with the lowest yields of all [1].

Rice production losses due to drought are common in more than 23 million ha of rain-fed area in South Asia, Southeast Asia, and Sub-Saharan Africa [5, 6]. In severe drought years, total rice production losses have reached as high as 40%, valued at US$650 million in the eastern Indian states of Jharkhand, Orissa, and Chhattisgarh alone [7]. In India, the 2002 drought affected 55% of the country's area and 300 million people when rice production declined by 20% from the interannual baseline [8]. Recent drought in 2009 has resulted in 16% reduction in rice production leading to high price rise and food security concerns. In 2004, a severe drought affected more than 2 million ha of the cropped area in Southeast Asia affecting the livelihood security of over 8 million people [8]. Drought also affects production in millions of hectares dependent on surface irrigation, where river flows and water impounded in ponds and reservoirs may be insufficient to irrigate the rice crop [9]. Water deficit is predicted to be a major challenge for sustainable rice production in future due to progressive climate change processes [100]. Despite drought being a major constraint to rice production, little success has been made in developing drought-tolerant rice cultivars. Most of the improved cultivars grown in drought-prone areas are the varieties originally bred for irrigated conditions and are highly susceptible to drought. Predicted increase in incidence of drought due to climate change presses the need to develop drought-tolerant high-yielding cultivars [10–12].

Drought may simply be defined as reduction in yield due to shortage of water [13]. Drought in the rice crop is classified on the basis of growth stage of the crop facing drought. There may be four kinds of drought: (i) seedling stage drought, (ii) vegetative stage drought, (iii) intermittent drought, and (iv) reproductive stage or terminal drought. Reproductive stage drought is the most devastating in terms of yield reduction [14]. Drought tolerance has long been regarded as a complex trait related to various physiological and biochemical parameters. These may include root traits, osmotic adjustment (OA), and maintenance of plant water status. QTL for these component traits and yield under drought have been identified and are being employed in molecular breeding of rice.

31.2
Morphophysiological Basis and Breeding for Drought Tolerance in Rice

Rice plant employs several mechanisms to deal with the drought stress and these mechanisms vary widely from one cultivar to other. Plants may adopt shorter life cycle to escape or avoid drought or they may have phenological adaptations, for example, thick cuticles for reduced evapotranspiration and long and thick roots to enable the plant to fetch water from deeper soil layers. Physiological traits such as osmotic adjustment to maintain cell turgor pressure during dehydration and ability to recover from desiccation are also important components of drought tolerance [15, 16]. Regarding traits for breeding drought tolerance in rice, there are two important considerations, the stress responsiveness of the trait and maintenance of plant water status (Figure 31.1, [17]).

Rice is a highly drought-susceptible plant. One of the reasons for this is its very thin layer of epicuticular wax that is about 20% of that in sorghum, a relatively drought-tolerant crop [15]. The resistance of rice cuticle to water loss is, therefore, low and it loses water even when its stomata are closed [18, 19]. Upland rice cultivars that are relatively more drought tolerant usually have a thicker epicuticular wax layer than the irrigated rice cultivars, indicating that the wax layer plays an important role in drought resistance. Osmotic adjustment is another well-known phenomenon that enables plants to survive water stress, but application of this trait in rice breeding is still under debate. Some scientists believe that it can be an important part of the solution leading to development of drought-tolerant rice [15, 20], whereas others are skeptical about the usefulness of this approach [18].

In upland varieties, a deeper root system enables rice plants to extract more water from the lower soil layers under drought conditions [21]. In most cases, the number of roots may not matter, but thickness and length of the roots help large xylem vessels to extract moisture even under severe stress [15, 18]. Root-to-shoot weight ratio becomes quite important in this case. Higher the root-to-shoot weight ratio, the more a rice cultivar is likely to be tolerant to drought. However, partitioning of carbon between source (shoot) and sink is important here because if too much carbon is utilized for the root growth, then yield is likely to be adversely affected [22].

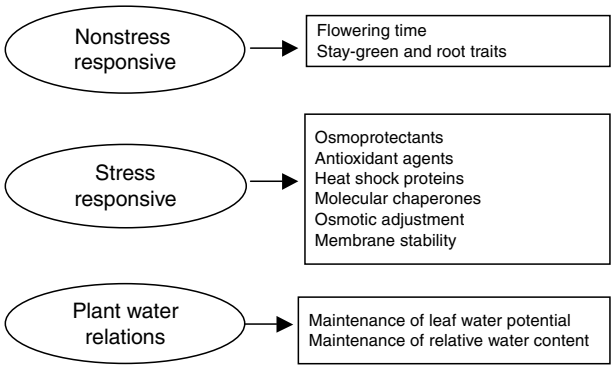

Figure 31.1 Different categories of drought tolerance traits in rice.

Lack of effective selection criteria for component traits of drought tolerance and low heritability of grain yield under stress are cited as major reasons for the slow progress in breeding for drought tolerance and the use of secondary traits for yield improvement [23–31]. Gains in yield by selecting for secondary traits have not yet been clearly demonstrated in rice. Significant scientific progress over the last 6 years in (i) stress genomics, (ii) breeding and phenotyping methodology [12, 32, 33], and (iii) better understanding of the drought tolerance mechanisms [18] has made it promising to develop drought-tolerant varieties with high yield potential. Recent studies at International Rice Research Institute (IRRI) have demonstrated the effectiveness of direct selection for grain yield under drought stress [12, 13, 34–36]. Direct selection for grain yield under drought has led to release of two breeding lines developed at IRRI, namely, IR74371-70-1-1 and IR74371-54-1-1, for cultivation in India and Philippines, respectively, in 2009.

Breeding line IR74371-70-1-1 was developed at IRRI from IR55419-04*2/Way Rarem cross, IR55419-04 being the drought-tolerant donor, and distributed to National Agricultural Research and Education Systems (NARES) partners in 2003. A selection from this line, IR 74371-70-1-1-CRR-1, was first tested at several locations in IRRI-India Upland Rice Shuttle Breeding Network (URSBN). It was nominated for testing under the All India Coordinated Rice Improvement Program (AICRIP) by Central Rainfed Upland Rice Research Station (CRURRS) of ICAR from 2005 to 2007. In the AICRIP trials, it showed yield advantage of 29.2 and 19.1% over the national and regional check varieties under drought-affected situations. It has an average yield advantage of 0.5 ton ha^{-1} under moderate drought and 1.0 ton ha^{-1} under severe drought over IR64. It was released by the name "Sahbhagi Dhan" for cultivation in drought-prone Jharkhand and Orissa states of India.

31.3 Mapping of QTL for Drought Tolerance in Rice

Quantitative trait loci (QTL) have been identified for almost all drought-related traits in rice. Around 20 different mapping populations have been screened both for drought-related secondary traits and for yield under drought (Table 31.1). Slow progress in breeding for drought-tolerant rice varieties has been attributed to failure to identify QTL with large and consistent effects on yield that could be used for marker-assisted selection [16, 37–42]. Most of the mapping populations studied for the identification of secondary traits associated with drought tolerance have been derived from parents that do not differ very widely for drought tolerance. Many traditional varieties and land races of rice adapted to drought-prone areas have high level of drought tolerance, but they have rarely been used as parents in the QTL mapping studies. Many of the mapping populations used for QTL mapping are derived from indica/japonica crosses because they show higher marker polymorphism [43].

Recent research at IRRI in partnership with NARES using well-characterized drought-tolerant donors as one of the parents in the mapping/breeding populations

Table 31.1 Different traits and mapping populations used for the identification of drought tolerance QTL in rice.

Trait	Population	References
Osmotic adjustment, dehydration tolerance	CO39/Moroberekan	[44]
Root morphology	Azucena/Bala	[22]
Leaf rolling, stomatal conductance	Azucena/Bala	[22]
Root morphology, root distribution	IR64/Azucena	[41]
Tiller number, total/penetrated roots ratio	Azucena/Bala	[45]
CMS under drought	CT9993/IR62266	[40]
Root characteristics	IR58821/IR52561	[46]
Root thickness, root penetration index	IR64/Azucena	[42]
Leaf rolling, leaf drying, RWC, growth rate	IR64/Azucena	[47]
Drought-related morphological and physiological traits	IR64/Azucena	[38]
Root traits, OA	CT9993/IR62266	[16]
Root growth, dehydration avoidance	Azucena/Bala	[48]
Root traits, shoot biomass	CT9993/IR62266, IR58821/IR52561	[39]
Yield and root traits under drought	IR64/Azucena	[49]
Stay green character, chlorophyll content	Hwacheong-wr mutants	[50]
Yield, biomass, OA, root traits	CT9993/IR62266	[37]
Root traits	IR1552/Azucena	[51]
Yield components under drought	Bala/Azucena	[52]
Yield components under drought	Introgression lines	[53]
Dehydration avoidance	Indica × Japonica	[54]
Dehydration avoidance	*O. rufipogon* introgression lines	[55]
Dehydration avoidance	IR58821/IR52561	[56]
Root length (constitutive)	Kalinga III/Azucena	[57]
Yield under lowland drought stress	CT9993/IR62266	[58]
Yield under drought at reproductive stage	Vandana/Way Rarem	[59]
Morphophysiological traits, yield under drought	Taking background	[60]
Growth and development under PEG stress	Akihikari/IRAT109	[61]
Leaf epicuticular wax and other trait responses under drought	CT9993-5-10-1-M/IR62266-42-6-2	[62]
Yield under drought at reproductive stage	N22/Swarna, N22/IR64, N22/MTU1010	[63, 64]
Yield under drought at reproductive stage	Apo/Swarna	[65]
Drought resistance traits	IR20/Nootripathu	[66]

has identified QTL with major and consistent effect on rice yield under drought [58, 59, 65]. Bernier *et al*. reported a QTL on chromosome 12 (q$DTY_{12.1}$) in the Vandna/Way Rarem population that explained 51% of the genetic variance for yield under drought [59]. Till now this QTL has shown the largest effect for grain yield under drought in several genetic backgrounds. The q$DTY_{12.1}$ also showed large and consistent effect in target environments in a wide range of moderate to severe drought situations [67, 68]. A major QTL for grain yield under lowland drought, explaining 32% of the phenotypic variance, was reported on rice chromosome 1 in CT9993/IR62266 population [58]. Subsequent studies have identified major QTL with consistent effect on grain yield under lowland drought on chromosome 2 (q$DTY_{2.1}$) and 3 (q$DTY_{3.1}$) in Apo/2*Swarna mapping population. The $DTY_{2.1}$ and $DTY_{3.1}$ QTL explain 13–16 and 31% of the phenotypic variance of the trait, respectively, rendering them useful for marker-assisted breeding for yield under lowland drought stress [65]. Recently, a common QTL for yield under drought (q*DTY1.1*) has been reported in three different mapping populations involving Indian drought-tolerant variety N22 [63, 64].

In case of new rice for Africa (NERICA), drought tolerance QTL have been introduced from *Oryza glaberrima* into *O. sativa*, producing better-adapted alien introgression lines for drought-prone areas of African continent [48]. QTL for yield have also been identified in *O. rufipogon* and other wild rice species that may provide a source of new genes for drought tolerance [45, 48, 69, 70]. This approach also opens up opportunities for the application of genomics for the identification of drought tolerance genes. Comparative genome analysis shows that some of the drought tolerance QTL identified in rice have their homologues in other crop species, for example, barley and maize, indicating that genes conferring drought tolerance in one grass species may have a similar effect on another species of this family. These genes seem to be conserved in many different grass species during the course of evolution; therefore, knowledge gained from the research carried out in rice will be useful in breeding other cereal crops and vice versa [16].

31.4 Meta-Analysis of Drought Tolerance QTL in Rice

A large number of minor QTL for different drought-related traits have been mapped on almost all the 12 rice chromosomes. Therefore, the use of bioinformatics tools has become imperative for the identification of consensus QTL and candidate genes. Meta-analysis combines the results of several QTL mapping studies and provides narrow confidence intervals for meta-QTL. This simplifies the identification and positional cloning of the candidate genes. Meta-analysis is usually applied to multiple mapping populations, but it can be applied to a single population as well [71]. Combining QTL data from studies employing different mapping populations would be extremely helpful in identifying candidate genes by positioning consistent QTL with more precision. Meta-analysis of QTL enables us to work out "hot spot" regions in the genome. Within those regions, one can look for the gene(s) underlying QTL

more precisely. This approach has already been applied for the analysis of root trait QTL in rice [72]. Interestingly, a QTL mapped on the long arm of chromosome 1 for grain yield under drought in different populations emerged as one of the hot spots for the root trait QTL in the meta-analysis [72].

31.5 Marker-Assisted Selection and Pyramiding of Drought Tolerance QTL in Elite Rice Cultivars

A large number of QTL have been identified in rice for drought tolerance and yield under drought, but their introgression in the popular varieties of rice has just begun. The major drawback in this approach is the linkage drag – transfer of undesired traits along with the trait of interest due to their tight genetic linkage. One of the most successful examples of marker-assisted backcross breeding in rice for abiotic stress tolerance is introgression of *Sub1* gene for submergence tolerance into popular mega variety of rice "Swarna" to create Swarna-*sub1*, where three kinds of markers were used: (i) a gene-based functional marker for the selection of favorable *Sub1* allele, (ii) two markers flanking the QTL to eliminate the linkage drag, and (iii) random background markers for fast recovery of the genetic background of the recipient variety during backcrossing [73]. Drought-tolerant donors in most populations screened for the QTL analysis are traditional varieties with low-yield potential, poor response to high-input management, early duration, taller plants, and sometimes carrying undesirable characteristics such as high grain shattering. One or more of such undesirable traits may be located near the drought QTL regions, hence a potential linkage drag in breeding for drought tolerance.

Despite the large number of drought tolerance QTL identified, limited attempts have been made for the introgression of these QTL into high-yield breeding lines [43, 69]. The limited success in the past may be due to involvement of minor QTL explaining very small proportion of the total phenotypic variation (5.6–17.7%) and lack of adequate fine mapping to develop tightly linked markers for breeding applications. As a result, the desirable genes could be lost due to recombination between gene for the trait and the marker during backcrossing [74]. Recent progress in identification and fine-mapping of major QTL for yield under drought has paved the way for introgression of such QTL in mega rice varieties through marker-assisted backcrossing in the near future.

Marker-assisted selection (MAS) could be applied with some modification of strategies such as single large-scale marker-assisted selection (SLS-MAS) and marker-assisted recurrent selection (MARS) [75, 76]. The main features of SLS-MAS are that (i) MAS could be performed at F2 or preferably F3 populations derived from elite lines, (ii) flanking markers are less than 5 cM away from the QTL, and (iii) QTL chosen should explain large proportion of the phenotypic variation and be stable across environments. This involves a four-step procedure: (i) identification of elite lines outstanding for the trait of interest, (ii) identification of the most favorable genomic regions for each parental line, (iii) intercrossing of elite lines to develop

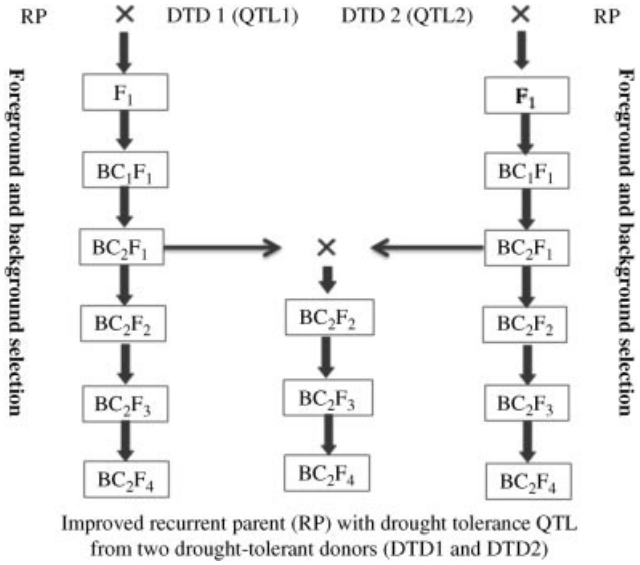

Figure 31.2 Schematic plan for simultaneous but stepwise method for transfer of drought tolerance QTL/genes from multiple donors in rice (A. K. Singh, Genetics, IARI, New Delhi).

segregating populations, and (iv) selection of plants homozygous for the favorable alleles at target loci.

Pyramiding of QTL is another approach for breeding drought-tolerant rice. A large number of QTL introgression lines (ILs) could be created in elite backgrounds using backcross (BC) breeding, each of which carries different genomic segments for improved drought tolerance from known donors. The ILs are then intercrossed to pyramid all QTL into one variety (Figure 31.2). A drawback of this approach is the linkage drag from donor parents used for the introgression of different QTL. Therefore, this needs to be approached with utmost care, particularly when targeting introgression of several QTL into one line [77].

31.6 Comparative Genomics for Drought Tolerance

Comparative mapping of the genomic regions across species is an interesting alternative for the identification and positional cloning of the candidate genes underlying drought tolerance QTL. QTL for leaf water potential, relative water content, and other drought-related traits have been identified on barley chromosome 7 H [78]. Leaf water potential and RWC QTL are also identified at orthologous position on rice chromosome 8 in the C039/Moroberekkan mapping population [44]. A major gene called *Or* controlling osmoregulation has been identified at the same orthologous position in wheat chromosome 7A [79]. The synteny and colinearity of genes among cereals is much more complex with many exceptions, and it is difficult

to find comparable QTL map locations in cereals due to polyploidy and transposition of genes [80]. However, such analysis is possible by microsynteny analysis for similar QTL regions in different cereals as shown in the above example of the *Or* gene.

31.7 Transcriptomics and Proteomics for the Identification of Drought Tolerance Genes in Rice

Since hot spots for drought tolerance QTL in the rice genome are known, it is now relatively simple to identify differentially expressed candidate genes in these regions for understanding drought tolerance in rice. *In silico* analyses have been carried out for the QTL regions flanked by the molecular markers and candidate genes identified through functional homology. A study has identified 48 candidate genes on rice chromosome 1 between markers RM212 and RM319, of which 16 were suggested for their potential role in drought tolerance [81]. Similar *in silico* analyses have been done and candidate genes identified on chromosome 1, 2, 4, 8, and 9 [82]. Several transcription factor genes, for example, *CBF/DREB1*, *DREB2*, *RD29B*, *RD22*, *ICE1*, *CDPK*, *ABF3*, *CBF3*, and *SNAC1* have been studied for their differential expression and regulatory role under drought stress [83–86]. Some of these genes have been transferred to rice through transgenic approach to validate their role in drought stress tolerance (Table 31.2).

Table 31.2 List of transcription factors genetically transformed in rice for drought tolerance.

Gene	Trait	References
HVA1 (barley group 3 LEA protein)	Drought and salt	[87]
P5CS, encoding pyrroline-5-carboxylate synthetase	Drought	[88]
OsCDPK7 (rice calcium-dependent protein kinase)	Cold, drought, salt	[85]
TPS (trehalose-6-phosphate synthase), *TP* (trehalose-6-phosphatase)	Drought	[89–91]
Dadc (*D. stramonium* arginine decarboxylase)	Drought	[92]
RWC3	Drought	[93]
ABF3 (*Arabidopsis* ABRE-binding factor 3)	Drought	[84]
DREB1A (*Arabidopsis* DRE-binding protein 1)	Drought and salt	[84]
MnSOD (pea Mn superoxide dismutase)	Drought	[94]
SNAC1 (rice stress-responsive NAC1)	Drought and salt	[83]
OsDREB1 (rice DRE binding protein 1)	Drought, salt, cold	[95]
HvCBF4 (barley C-repeat binding factor)	Drought, salt, cold	[96]
OsCIPK12 (rice calcineurin B-like protein-interacting protein kinase 12)	Drought	[97]
OCPI1	Drought	[98]
ZFP252 (rice TFIIIA-type zinc finger protein)	Drought, salt	[99]
OsDHODH1	Drought, salt	[100]
ONAC045 (NAC gene)	Drought, salt	[101]

Most of the genes presumed to be involved in the drought tolerance are involved in (i) signal transduction, (ii) osmotic adjustment, and (iii) transcriptional regulation of the stress response pathway, for example, *DREB1* and *SNAC1*. Transgenic rice with overexpression of *SNAC1* gene showed 22–34% higher seed setting than control plants [83]. However, successful commercialization of these transgenic lines is still questionable due to complex gene interactions. Despite considerable progress in the development of transgenic rice lines, their impact on enhancing drought tolerance under field conditions is still awaited. These lines need to be tested in field drought environments and for their biosafety and other regulatory issues prior to their deployment in popular rice varieties through marker-assisted backcross breeding. Screening of transgenic plants for drought tolerance has been usually done under controlled glasshouse, but selection for drought-tolerant rice varieties is advocated in the target drought-prone environments [102, 103].

Proteomics approach has also been followed by some workers, and more than 2000 proteins were analyzed by 2D electrophoresis [104]. Drought-induced changes were observed in actin depolymerizing factor (ADF), which is a chloroplastic glutathione-dependent dehydroascorbate reductase. ADF concentration was higher in drought-tolerant cultivars before stress and it increased further in leaf blades, leaf sheaths, and roots after exposure to drought, suggesting that ADF could be one of the target proteins for drought tolerance [105]. Differentially expressed proteins could be worked out through protein profiling of the mapping populations.

31.8 Conclusions

Rice is a semiaquatic species highly susceptible to drought. Global warming, unpredictable rainfall patterns, and climate change would cause more severe stress situations in the rain-fed agriecosystems in future. Efforts for developing drought-tolerant rice varieties through conventional breeding by direct selection for grain yield under drought have yielded some success, and molecular breeding approaches are now beginning to be employed for introgression of major QTL for grain yield under drought. Genes underlying these QTL are being deciphered using genomics approaches. Transgenic rice plants have also been developed with transcription factors such as *DREB1* and *SNAC1*, but these need to be evaluated under field drought environment to validate their actual potential for increasing rice production. However, drought tolerance is a complex trait and discovery of useful alleles of the genes involved in the drought response pathway requires extensive use of genomics approaches and high-precision phenotyping employing modern phenomic facilities in addition to repeated field phenotyping in the target environment.

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References

1 Khush, G.S. (1997) Origin, dispersal, cultivation and variation of rice. *Plant Mol. Biol.*, **35**, 25–34.

2 Asia Rice Foundation: News on Rice (dated - September 23, 2004) Available at http://www.asiarice.org/sections/chapters/Philippines/ARF-Phil-About.html (accessed 8 April 2005).

3 United Nations (2002) World population prospects: the 2002 revision. p. 36. Available at http://www.un.org/esa/population/publications/wpp2002/. (accessed 5 December 2004).

4 Poehlman, J.M. and Sleper, D.A. (1995) *Breeding Field Crops*, 4th edn, Iowa State Press, p. 494.

5 Huke, R.E. and Huke, E.H. (1997) *Rice area by type of culture: South, Southeast, and East Asia*, IRRI, Los Baños, Philippines.

6 Monty, J. (2009) The prospects for doubling rice production in Africa in 10 years. Paper presented at 14th Australian Plant Breeding and 11th SABRAO conference organized by Australasian Plant Breeding Conference and Society for the Advancement in Breeding Research in Asia and Oceania Congress in Cairns, Australia from August 10–14, 2009.

7 Pandey, S., Bhandari, H.N., Sharan, R., Naik, D., Taunk, S.K., and Sastri, A.D.R.A.S. (2005) *Economic Costs of Drought and Rainfed Rice Farmers' Coping Mechanisms in Eastern India: Final Project Report*. International Rice Research Institute, Manila, Philippines.

8 Pandey, S., Bhandari, H.N., and Hardy, B. (2007) *Economic Costs of Drought and Rice Farmers' Coping Mechanisms*, IRRI, Los Baños, Philippines.

9 Maclean, J.L., Dawe, D.C., Hardy, B. and Hettel, G.P. (eds) (2002) *Rice Almanac. Los Baños (Philippines); International Rice Research Institute, Bouaké (Côte d'Ivoire) West Africa Rice Development Association, Cali (Colombia); International Center for Tropical Agriculture, Rome (Italy)*, Food and Agriculture Organization., p. 253.

10 Wassmann, R., Jagadish, S.V.K., Sumfleth, K., Pathak, H., Howell, G., Ismail, A., Serraj, R., Redoña, E., Singh, R.K., and Heuer, S. (2009) Regional vulnerability of climate change impacts on Asian rice production and scope for adaptation. *Adv. Agron.*, **102**, 91–133.

11 Bates, B.C., Kundzewicz, Z.W., Wu, S., and Palutikof, J.P. (eds) (2008) *Climate Change and Water: Technical Paper of the Intergovernmental Panel on Climate Change*, IPCC Secretariat, Geneva, p. 210. Available at http://www.ipcc.ch/ipccreports/tp-climate-change-water.htm. (accessed 1 July 2009).

12 Kumar, A., Bernier, J., Verulkar, S., Lafitte, H.R., and Atlin, G.N. (2008) Breeding for drought tolerance: direct selection for yield, response to selection and use of drought-tolerant donors in upland and lowland-adapted populations. *Field Crops Res.*, **107**, 221–231.

13 Bernier, J., Atlin, G.N., Serraj, R., Kumar, A., and Spaner, D. (2008) Breeding upland rice for drought resistance. *J. Sci. Food Agric.*, **88**, 927–939.

14 Kamoshita, A., Babu, R.C., Boopathi, N.M., and Fukai, S. (2008) Phenotypic and genotypic analysis of drought-resistance traits for development of rice cultivars adapted to rainfed environments. *Field Crops Research*, **109**, 1–23.

15 Nguyen, H.T., Babu, R.C., and Blum, A. (1997) Breeding for drought resistance in rice: physiology and molecular genetics considerations. *Crop Sci.*, **37**, 1426–1434.

16 Zhang, J., Zheng, H.G., Aarti, A., Pantuwan, G., Nguyen, T.T., Tripathy, J.N., Sarial, A.K., Robin, S., Babu, R.C., Nguyen, B.D., Sarkarung, S., Blum, A., and Nguyen, H.T. (2001) Locating genomic regions associated with components of drought resistance in rice: comparative mapping within and across species. *Theor. Appl. Genet.*, **103**, 19–29.

17 Blum, A. (2002) Drought tolerance: is it a complex trait? in *Field Screening for Drought Tolerance in Crop Plants with Special Emphasis on Rice: Proceedings of an International Workshop on Field Screening for Drought Tolerance in Rice, 11–14 December, 2000, International Crop Research Institute for Semi-arid Tropics, Patancheru, India. Patancheru, India* (eds N.P. Saxena and J.C. O'Toole),

International Crop Research Institute for Semi-arid Tropics, Rockefeller Foundation, New York, USA, pp. 17–24.
18 Fukai, S. and Cooper, M. (1995) Development of drought-resistant cultivars using physio-morphological traits in rice. *Fields Crop Res.*, **40**, 67–86.
19 Haque, M.M., Mackill, D.J., and Ingram, K.T. (1992) Inheritance of leaf epicuticular wax content in rice. *Crop Sci.*, **32**, 865–868.
20 Babu, R.C., Shashidhar, H.E., Lilley, J.M., Thanh, N.D., Ray, J.D., Sadasivam, S., Sarkarung, S., O'Toole, J.C., and Nguyen, H.T. (2001) Variation in root penetration ability, osmotic adjustment and dehydration tolerance among accessions of rice to rainfed lowland and upland ecosystems. *Plant Breed.*, **120**, 233–238.
21 Mambani, B. and Lal, R. (1983) Response of upland rice varieties to drought stress. II. Screening rice varieties by means of variable moisture regimes along a toposequence. *Plant Soil*, **73**, 73–94.
22 Price, A.H. and Tomos, A.D. (1997) Genetic dissection of root growth in rice (*Oryza sativa* L.). II: mapping quantitative trait loci using molecular markers. *Theor. Appl. Genet.*, **95**, 143–152.
23 Blum, A. (1988) *Plant Breeding for Stress Environments*, CRC Press, Boca Raton, FL.
24 Edmeades, G.O., Bolanos, J., Lafitte, H.R., Rajaram, S., Pfeiffer, W., and Fischer, R.A. (1989) Traditional approaches to breeding for drought resistance in cereals, in *Drought Resistance in Cereals* (ed. F.W.G. Baker), CAB International, Wallingford, Oxon, UK, pp. 27–52.
25 Fukai, S., Pantuwan, G., Jongdee, B., and Cooper, M. (1999) Screening for drought resistance in rainfed lowland rice. *Field Crops Res.*, **64**, 61–74.
26 Jongdee, B., Fukai, S., and Cooper, M. (2002) Leaf water potential and osmotic adjustment as physiological traits to improve drought tolerance in rice. *Field Crops Res.*, **76**, 153–163.
27 Ouk, M., Basnayake, J., Tsubo, M., Fukai, S., Fischer, K.S., Cooper, M., and Nesbitt, H. (2006) Use of drought response index for identification of drought tolerant genotypes in rainfed lowland rice. *Field Crops Res.*, **99**, 48–58.
28 Pantuwan, G., Fukai, S., Cooper, M., Rajatasereekul, S., and O'Toole, J.C. (2002) Yield response of rice (*Oryza sativa* L.) to drought under rainfed lowlands. 3. Plant factors contributing to drought resistance. *Field Crops Res.*, **73**, 181–200.
29 Price, A. and Courtois, B. (1999) Mapping QTLs associated with drought resistance in rice: progress, problems, and prospects. *Plant Growth Regul.*, **29**, 123–133.
30 Rosielle, A.A. and Hamblin, J. (1981) Theoretical aspects of selection for yield in stress and non-stress environments. *Crop Sci.*, **21**, 943–946.
31 Toorchi, M., Shashidhar, H.E., Gireesha, T.M., and Hittalmani, S. (2003) Performance of backcross involving transgressant doubled haploid lines in rice under contrasting moisture regimes: yield components and marker heterozygosity. *Crop Sci.*, **43**, 1448–1456.
32 Atlin, G., Paris, T., and Courtois, B. (2002) Sources of variation in participatory varietal selection trials with rainfed rice: implications for the design of mother-baby trial networks, in *Quantitative Analysis of Data from Participatory Methods in Plant Breeding* (eds M.R. Bellon and J. Reeves), CIMMYT, Mexico, DF, pp. 36–43.
33 Jongdee, B., Pantuwan, G., Fukai, S., and Fischer, K. (2004) Improving drought tolerance in rainfed lowland rice: an example from Thailand, in *New Directions for a Diverse Planet (Proceedings of the 4th International Crop Science Congress, Brisbane Australia 26 September–1 October 2004)*, The Regional Institute Ltd, Gosford, New South Wales, Australia.
34 Atlin, G.N. and Lafitte, H.R. (2002) Marker-assisted breeding versus direct selection for drought tolerance in rice, in *Field Screening for Drought Tolerance in Crop Plants with Emphasis on Rice (Proceedings of an International Workshop on Field Screening for Drought Tolerance in Rice 11–14 Dec 2000)* (eds N.P. Saxena and J.C. O'Toole), ICRISAT, Patancheru, India, p. 208.
35 Venuprasad, R., Lafitte, H.R., and Atlin, G.N. (2007) Response to direct selection

for grain yield under drought stress in rice. *Crop Sci*., **47**, 285–293.

36 Venuprasad, R., Sta Cruz, M.T., Amante, M., Magbanua, R., Kumar, A., and Atlin, G.N. (2008) Response to two cycles of divergent selection for grain yield under drought stress in four rice breeding populations. *Field Crops Res*., **107**, 232–244.

37 Babu, R.C., Nguyen, B.D., Chamarerk, V., Shanmugasundaram, P., Chezhian, P., Jeyaprakash, P., Ganesh, S.K., Palchamy, A., Sadasivam, S., Sarkarung, S., Wade, L.J., and Nguyen, H.T. (2003) Genetic analysis of drought resistance in rice by molecular markers: association between secondary traits and field performance. *Crop Sci*., **43**, 1457–1469.

38 Hemamalini, G.S., Shashidhar, H.E., and Hittalmani, S. (2000) Molecular marker assisted tagging of morphological and physiological traits under two contrasting moisture regimes at peak vegetative stage in rice (*Oryza sativa* L.). *Euphytica*, **112**, 69–78.

39 Kamoshita, A., Zhang, J., Siopongco, J., Sarkarung, S., Nguyen, H.T., and Wade, L.J. (2002) Effects of phenotyping environment on identification of QTL for rice root morphology under anaerobic conditions. *Crop Sci*., **42**, 255–265.

40 Tripathy, J.N., Zhang, J., Robin, S., Nguyen, T.T., and Nguyen, H.T. (2000) QTLs for cell membrane stability mapped in rice (*Oryza sativa* L.) under drought stress. *Theor. Appl. Genet*., **100**, 1197–1202.

41 Yadav, R., Courtois, B., Huang, N., and McLaren, G. (1997) Mapping genes controlling root morphology and root distribution in a doubled haploid population of rice. *Theor. Appl. Genet*., **94**, 619–632

42 Zheng, H.G., Babu, R.C. Pathan, Md.M.S., Ali, L., Huang, N., Courtois, B., and Nguyen, H.T. (2000) Quantitative trait loci for root-penetration ability and root thickness in rice: comparison of genetic backgrounds. *Genome*, **43** 53–61.

43 Courtois, B., Shen, L., Petalcorin, W., Carandang, S., Mauleon, R., and Li, Z. (2003) Locating QTLs controlling constitutive root traits in the rice population IAC 165×Co39. *Euphytica*, **134**, 335–345.

44 Lilley, J., Ludlow, M., McCouch, S., and O'Toole, J.C. (1996) Locating QTL for osmotic adjustment and dehydration tolerance in rice. *J. Exp. Bot*., **47**, 1427–1436.

45 Price, A.H., Steele, K.A., Moore, B.J., Barraclough, P.B., and Clark, L.J. (2000) A combined RFLP and AFLP linkage map of upland rice (*Oryza sativa* L.) used to identify QTLs for root-penetration ability. *Theor. Appl. Genet*., **100**, 49–56.

46 Ali, M.L., Pathan, M.S., Zhang, J., Bai, G., Sarkarung, S., and Nguyen, H.T. (2000) Mapping QTLs for root traits in a recombinant inbred population from two indica ecotypes in rice. *Theor. Appl. Genet*., **101**, 756–766.

47 Courtois, B., McLaren, G., Sinha, P.K., Prasad, K., Yadav, R., and Shen, L. (2000) Mapping QTLs associated with drought avoidance in upland rice. *Mol. Breed*., **6**, 55–66.

48 Price, A.H., Steele, K.A., Moore, B.J., and Jones, R.G.W. (2002) Upland rice grown in soilfilled chambers and exposed to contrasting water-deficit regimes. II. Mapping quantitative trait loci for root morphology and distribution. *Field Crops Res*., **76**, 25–43.

49 Venuprasad, R., Shashidhar, H.E., Hittalmani, S., and Hemamalini, G.S. (2002) Tagging quantitative trait loci associated with grain yield and root morphological traits in rice (*Oryza sativa* L.) under contrasting moisture regimes. *Euphytica*, **128**, 293–300.

50 Cha, K.-W., Lee, Y.-J., Koh, H.-J., Nam, Y.-W., and Paek, N.-C. (2002) Isolation, characterization and mapping of stay green mutant in rice. *Theor. Appl. Genet*., **104**, 526–532.

51 Zheng, B.S., Yang, L., Zhang, W.P., Mao, C.Z., Wu, Y.R., Yi, K.K., Liu, F.Y., and Wu, P. (2003) Mapping QTLs and candidate genes for rice root traits under different water supply conditions and comparative analysis across three populations. *Theor. Appl. Genet*., **107**, 1505–1515.

52 Lafitte, H.R., Price, A.H., and Courtois, B. (2004) Yield response to water deficit in an upland rice mapping population:

associations among traits and genetic markers. *Theor. Appl. Genet.*, **109**, 1237–1246.

53 Xu, J.L., Lafitte, H.R., Gao, Y.M., Fu, B.Y., Torres, R., and Li, Z.K. (2005) QTLs for drought escape and tolerance identified in a set of random introgression lines of rice. *Theor. Appl. Genet.*, **111**, 1642–1650.

54 Yue, B., Xue, W., Xiong, L., Yu, Z., Luo, L., Cui, K., Jin, D., Xing, Y., and Zhang, Q. (2006) Genetic basis of drought resistance at reproductive stage in rice: separation of drought resistance from drought avoidance. *Genetics*, **172**, 1213–1228.

55 Xia, Z., Shaoxia, Z., Yongcai, F., Zhen, S., Xiangcun, W., and Chuanquin, S. (2006) Identification of a drought tolerant introgression line derived from Dongxiang common wild rice (*O. rufipogon* Griff.). *Plant Mol. Biol.*, **62** (1–2), 247–259.

56 Manickavelu, A., Nadarajan, N., Ganesh, S.K., Gnanamalar, R.P., and Babu, R.C. (2006) Drought tolerance in rice: morphological and molecular genetic consideration. *Plant Growth Regul.*, **50** (2–3), 121–138.

57 Steel, K.A., Price, A.H., Shashidhar, H.E., and Witcombe, J.R. (2006) Marker-assisted selection to introgress rice QTLs controlling root traits into an Indian upland rice variety. *Theor. Appl. Genet.*, **112**, 208–221.

58 Kumar, R., Venuprasad, R., and Atlin, G. (2007) Genetic analysis of rainfed lowland rice drought tolerance under naturally occurring stress in Eastern India: heritability and QTL effects. *Field Crops Res.*, **103**, 42–52.

59 Bernier, J., Kumar, A., Ramaiah, V., Spaner, D., and Atlin, G.N. (2007) A large-effect QTL for grain yield under reproductive-stage drought stress in upland rice. *Crop Sci.*, **47**, 507–518.

60 Zhao, X.-Q., Xu, J.-L., Zhao, M., Lafitte, R., Zhu, L.-H., Fu, B.-Y., Gao, Y.-M., and Li, Z.-K. (2008) QTLs affecting morph-physiological traits related to drought tolerance detected in overlapping introgression lines of rice (*Oryza sativa* L.). *Plant Sci.*, **174** (6), 618–625.

61 Kato, Y., Hirotsu, S., Nemoto, K., and Yamagishi, J. (2008) Identification of QTLs controlling rice drought tolerance at seedling stage in hydroponic culture. *Euphytica*, **160** (3), 423–430.

62 Srinivasan, S., Gomez, M.S., Kumar, S.S., Ganesh, S.K., Biji, K.R., Senthil, A., and Babu, R.C. (2008) QTLs linked to leaf epicuticular wax, physio-morphological and plant production traits under drought stress in rice (*Oryza sativa* L.). *Plant Growth Regul.*, **56**, 245–256.

63 Vikram, P., Kumar, A., Dixit, S., Espirtu, M., and Cruz, T.S. (2009) Comparative molecular approaches to identify large effect drought tolerant QTLs in rice, 11–16 October 2009, Shanghai, China, p. 214.

64 Vikram, P., Sta Cruz, M.T., Espiritu, M., Del Valle, M., Singh, A.K., and Kumar, A. (2009) Major QTLs for grain yield under drought in the background of mega varieties. 6th International Rice Genetics Symposium, 16–19 November 2009, Manila Hotel, Manila, Philippines, p. 79.

65 Venuprasad, R., Dalid, C.O., Del Valle, M., Zhao, D., Espiritu, M., Sta Cruz, M.T., Amante, M., Kumar, A., and Atlin, G.N. (2009) Identification and characterization of large-effect quantitative trait loci for grain yield under lowland drought stress in rice using bulk-segregant analysis. *Theor. Appl. Genet.*, **120**, 177–190.

66 Gomez, S.M., Manikanda, N.B., Kumar, S.S., Ramasubramanian, T., Chengsong, Z., Jeyaprakash, P., Senthil, A., and Babu, R.C. (2009) Molecular mapping and location of QTLs for drought-resistance traits in *indica* rice (*Oryza sativa* L.) lines adapted to target environments. *Acta Physiol. Plant.* doi: 10.1007/s11738-009-0413-1

67 Bernier, J., Serraj, R., Kumar, A., Venuprasad, R., Impa, S., Gowda, R.P., Oane, R., Spaner, D., and Atlin, G.N. (2009) The large-effect drought-resistance QTL qtl12.1 increases water uptake in upland rice. *Field Crops Res.*, **110**, 139–146.

68 Bernier, J., Kumar, A., Spaner, D., Verulkar, S., Mandal, N.P., Sinha, P.K.,

Peeraju, P., Dongre, P.R., Mahto, R.N., and Atlin, G.N. (2009) Characterization of the effect of rice drought tolerance *qtl12.1* over a range of environments in the Philippines and Eastern India. *Euphytica*, **166**, 207–217.

69 Price, A.H. (2002) QTLs for root growth and drought resistance in rice, in *Molecular Techniques in Crop Improvement* (eds S.D. Jain, D.S. Brar, and B.S. Ahoowalia), Kluwer, pp. 563–584.

70 Price, A.H., Townend, J., Jonnes, M.P., Audebert, A., and Courtois, B. (2002) Mapping QTLs associated with drought avoidance in upland rice grown in Philippines and West Africa. *Plant Mol. Biol.*, **48** (5–6), 683–695.

71 Khowaja, S.F., Gareth, N.J., Courtois, B., and Price, A.H. (2009) Improved resolution in the position of drought-related QTLs in a single mapping population of rice by meta-analysis. *BMC Genomics*, **10**, 276.

72 Courtois, B., Ahmadi, N., Khowaja, F., Price, A.H., Rami, J.-F., Frouin, J., Hamelin, C., and Ruiz, M. (2009) Rice root genetic architecture: meta-analysis from a drought QTL database. *Rice*, **2**, 115–128.

73 Neeraja, C.N., Maghirang-Rodriguez, R., Pamplona, A., Heuer, S., Collard, B.C.Y., Septiningsih, E.M., Vergara, G., Sanchez, D., Xu, K., Ismail, A.M., and Mackill, D.J. (2007) A marker-assisted backcross approach for developing submergence-tolerant rice cultivars. *Theor. Appl. Genet.*, **115**, 767–776.

74 Shen, L., Courtois, B., McNally, K., Robin, S., and Li, Z. (2001) Evaluation of near-isogenic lines of rice introgressed with QTLs for root depth through marker-aided selection. *Theor. Appl. Genet.*, **103**, 75–83.

75 Ribaut, J.-M. and Betr'an, J. (1999) Single large scale-marker assisted selection (SLS-MAS). *Mol. Breed.*, **5**, 531–541.

76 Mayor, P.J. and Bernardo, R. (2009) Genomewide selection and marker-assisted recurrent selection in double haploid verses F2 populations. *Crop Sci.*, **49**, 1719–1725.

77 Zikang, L., Dwivedi, D., Gao, Y.M., Zheng, T.Q., Laffitte, R., Xu, J.L., Mackill, D., Fu, B.Y., Domingo, J., Sun, Y., and Zhu, L.H. (2007) Improving drought tolerance of rice by designed QTL pyramiding. *Mol. Plant Breed.*, **2**, 205–206.

78 Teulat, B., This, D., Khairallah, M., Borries, C., Ragot, C., Sourdille, P., Leroy, P., Monneveux, P., and Charrier, A. (1998) Several QTLs involved in osmotic-adjustment trait variation in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, **96**, 688–698

79 Morgan, J.M. and Tan, M.K. (1996) Chromosomal location of a wheat osmoregulation gene using RFLP analysis. *Aust. J. Plant Physiol.*, **23**, 803–806

80 ENSAM-INRA. Towards a Comparative Genomics of Drought Tolerance in Cereals: Lessons from a QTL Analysis in Barley, http://www.cimmyt.org/english/docs/proceedings/molecApproaches/pdfs/towars_comparative.pdf. (accessed 1 February 2009).

81 Wang, X.-S., Zhu, J., Mansueto, L., and Bruskiewich, R. (2005) Identification of candidate genes for drought stress tolerance in rice by the integration of a genetic (QTL) map with the rice genome physical map. *J. Zhejiang Univ. Sci.*, **6B** (5), 382–388.

82 Zeng, H., Zhong, Y., and Luo, L. (2006) Drought tolerance genes in rice. *Funct. Integr. Genomics*, **6**, 338–341.

83 Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., and Xiong, L. (2006) Overexpressing a NAM, ATAF and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci. USA*, **103**, 12987–12992.

84 Oh, S.-J., Song, S.I., Kim, Y.S., Jang, H.-J., Kim, S.Y., Kim, M. *et al.* (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiol.*, **138**, 341–351.

85 Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., and Izui, K. (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J.*, **23**, 319–327.

86 Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M. (2003) Regulatory network

of gene expression in the drought and cold stress responses. *Curr. Opin. Plant Biol.*, **6**, 410–417.

87 Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D., and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.*, **110**, 249–257.

88 Zhu, B., Su, J., Chang, M.C., Verma, D.P.S., Fan, Y.L., and Wu, R. (1998) Overexpression of a D1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water and salt stress in transgenic rice. *Plant Sci.*, **139**, 41–48.

89 Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochain, R.J., and Wu, R. (2002) Trehalose accumulation in rice plants confers high tolerance to different abiotic stresses. *Proc. Natl. Acad. Sci. USA*, **99**, 15898–15903.

90 Jang, I.C., Oh, S.J., Seo, J.S., Choi, W.B., Song, S.I., Kim, C.H., Kim, Y.S., Seo, H.S., Choi, Y.D., Nahm, B.H., and Kim, J.K. (2003) Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. *Plant Physiol.*, **131**, 516–524.

91 Lee, S.B., Kwon, H.B., Kwon, S.J., Park, S.C., Jeong, M.J., Han, S.E., Byun, M.O., and Daniell, H. (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. *Mol. Breed.*, **11**, 1–13.

92 Capell, T., Bassie, L., and Christou, P. (2004) Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. *Proc. Natl. Acad. Sci. USA*, **101**, 9909–9914.

93 Lian, H.L., Yu, X., Ye, Q., Ding, X.S., Kitagawa, Y., Kwak, S.S., Su, W.A., and Tang, Z.C. (2004) The role of aquaporin Hong-Li Lian, Xin Yu, Qin Ye, Xiao-Song Ding, Yoshichika Kitagawa, Sang-Soo Kwak, Wei-Ai Su, and Zhang-Cheng Tang RWC3 in drought avoidance in rice. *Plant Cell Physiol.*, **45**, 481–489.

94 Wang, F.-Z., Wang, Q.-B., Kwon, S.-Y., Kwak, S.-S., and Su, W.-A. (2005) Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. *J. Plant Physiol.*, **162**, 465–472.

95 Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M. *et al.* (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol.*, **47**, 141–153.

96 Oh, S.-J., Kwon, C.-W., Choi, D.-W., Song, S.I., and Kim, J.-K. (2007) Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice. *Plant Biotechnol. J.*, **5**, 646–656.

97 Xiang, Y., Huang, Y., and Xiong, L. (2007) Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. *Plant Physiol.*, **144**, 1416–1428.

98 Huang, Y., Xiao, B., and Xiong, L. (2007) Characterization of a stress responsive proteinase inhibitor gene with positive effect in improving drought resistance in rice. *Planta*, **226**, 73–85.

99 Xu, D.-Q., Huang, J., Guo, S.-Q., Yang, X., Bao, Y.-M., Tang, H.-J., and Zhang, H.-S. (2008) Overexpression of a TFIIIA-type zinc finger protein gene ZFP252 enhances drought and salt tolerance in rice (*Oryza sativa* L.). *FEBS Lett.*, **582**, 1037–1043.

100 Liu, W.-Y., Wang, M.-M., Huang, J., Tang, H.-J., Lan, H.-X., and Zhang, H.-S. (2009) The OsDHODH1 gene is involved in salt and drought tolerance in rice. *J. Integr. Plant Biol.*, **51** (9), 825–833.

101 Zheng, X., Chen, B., Lu, G., and Han, B. (2009) Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. *Biochem. Biophys. Res. Commun.*, **379**, 985–989.

102 Babu, R.C., Zhang, J., Blum, A., Ho, T.H.D., Wu, R., and Nguyen, H.T. (2004) HVA1: a LEA gene from barley confers dehydration tolerance in transgenic rice

(*Oryza sativa* L.) via cell membrane protection. *Plant Sci.*, **166**, 855–862.

103 Rabbani, M.A., Maruyama, K., Abe, H., Khan, A.A., Katsura, K., Ito, Y., Yoshiwara, K., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiol.*, **133**, 1755–1767.

104 Salekdeh, G.H. *et al.* (2002) A proteomic approach to analyzing drought- and salt-responsiveness in rice. *Field Crops Res.*, **76**, 199–219.

105 Ali, G.M. and Komatsu, S. (2006) Proteomic analysis of rice leaf sheath during drought stress. *J. Proteome Res.*, **5**, 396–403.

32
Rice: Improving Cold Stress Tolerance

Kodiveri Muthukalianan Gothandam

Rice plant normally grown in tropical and temperate climatic zones is often threatened by cold stress and is especially sensitive to chilling stress at the seedling and reproductive stages. Low temperature at the seedling stage can result in poor germination, stunted seedlings, yellowing or withering, and reduced tillering. Unpredictable cold snaps at the reproductive stage delay heading and result in pollen sterility, which was thought to be one of the key factors responsible for the reduction in grain yield of rice. Cold stress prevents the expression of full genetic potential of plants owing to its direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic, oxidative, and other stresses. In this chapter, various aspects of improving rice cold tolerance are discussed.

32.1
Introduction

Rice is one of the three important cereals and it is produced annually at worldwide levels of more than 600 million ton. Unlike the other major cereals, more than 90% of rice is consumed by humans. Approximately, half the world's population derives a significant proportion of their caloric intake from rice consumption. As little new land is available to increase rice cultivation, larger yields will be needed to meet the anticipated higher demand. On the other hand, temperature is a major factor that limits the geographical locations suitable for growing rice plants. Plants exhibit a maximum rate of growth and development at an optimum temperature or over a diurnal range of temperatures. When ambient temperature deviates from the optimal, physiological, biochemical, metabolic, and molecular changes occur within plants. This is an effort of plants to maximize growth and developmental processes and maintain cellular homeostasis during such adverse conditions. Under increasingly stressful conditions, plants experience progressively more abnormal, impaired, or dysfunctional cellular and whole-plant processes until the cardinal temperatures for survival are reached [1]. At the extremes of the natural temperature range of a plant, the degree of physiological, cellular, metabolic, and molecular dysfunction becomes so severe that it leads to death. Plants feel stress under both

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high and low temperature exposure. Mesophilic temperate plants possess inducible temperature stress tolerance. During high-temperature exposure, stress tolerance can be induced by exposure to short-term elevated temperature and this is known as acquired thermotolerance [2], while at lower temperatures, stress tolerance can be induced by exposure to reduced temperature and this is known as chilling tolerance and/or cold acclimation. Chilling tolerance is the ability of a plant to tolerate low temperatures (0–15 °C) without injury or damage [3], while cold acclimation is an enhanced tolerance to the physical and physicochemical vagaries of freezing stress [4]. Both cold acclimation and chilling tolerance involve an array of biochemical, molecular, and metabolic processes [2, 5, 6]. Exposure of plants to temperature stress leads to the modification of metabolism in two ways. First, plants try to adjust their cellular metabolism altered due to rising or falling of temperatures. Temperature stress changes the structure, catalytic properties, and function of enzymes [7] and membrane metabolite transporters. Interestingly, regulatory mechanisms of plants become active and function to restore normal metabolite levels and, most importantly, metabolic fluxes [8, 9]. Second, the modifications of metabolism in response to temperature stress are mainly linked to enhanced tolerance mechanisms. Many metabolites are thought to have important properties that could contribute to induced stress tolerance [10]. In this chapter, emphasis is placed on the general response to the cold stress, and various factors involved in cold acclimation and their roles in cold tolerance are discussed.

32.2 Effect of Cold Stress on Rice

Rice is widely cultivated in different natural environments. Compared to other cereal crops such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), rice is much more sensitive to low temperature as a result of its tropical origin [11]. For rice, temperatures lower than 20 °C decrease both the speed and the percentage of germination. Plants exposed to cold stress show various phenotypic symptoms that include reduced leaf expansion, wilting, and chlorosis (yellowing of leaves) and may lead to necrosis (death of tissue). The phenotypic symptoms of rice seedlings and mature plants upon exposure to cold stress are shown in Figure 32.1.

With regard to the genetic bases for cold tolerance in cultivated rice, few major genes, such as Chs1 [12], Cts1 [13], and Cts2 [14], are reported to confer cold tolerance at the seedling stage; however, recent quantitative trait locus (QTL) analyses revealed that numerous QTL for cold tolerance are present on the rice genome [15–21], suggesting that adaptation to cold climates might involve complex features in physiological and genetic mechanisms.

Cold stress also severely affects the reproductive development of plants and this has been seen in rice plants at the time of anthesis (floral opening), which leads to sterility in flowers. Male sterility is the most severe consequence among the many chilling-induced agronomic damages in rice production. The developmental stages from pollen formation to fertilization are the most vulnerable to low temperature through-

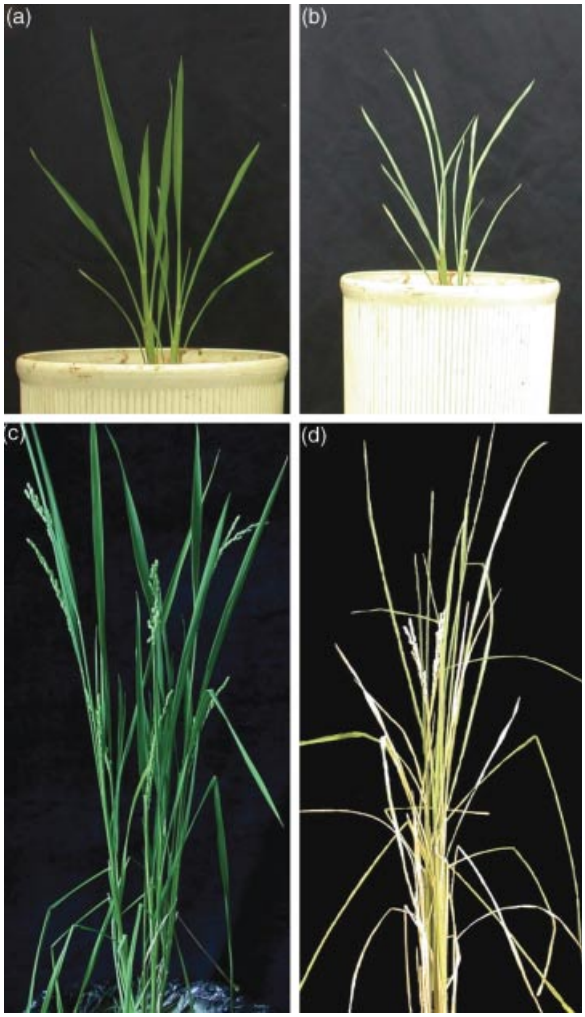

Figure 32.1 Effect of cold stress on rice seedlings and mature plant. Rice seedlings were exposed to 4 °C for 12 h and then allowed to recover. Mature rice plants were exposed to 4 °C for 36 h and allowed to recover. Damage due to cold stress is seen as wilting, chlorosis, and necrosis. (a and c) Unstressed. (b and d) Cold stressed.

out the life cycle of rice plants [22, 23]. It has been reported that the young microspore stage in pollen development was the most sensitive to low temperature [24]. Exposure of rice plants at the tetrad stage to a moderately low temperature (12 °C) for 4 days resulted in male sterility in 80% of spikelets [22, 25]. Microscopic observation of developing rice anthers suggested that one possible reason for the male sterility after low-temperature treatment was the failure of anther development. The observed abnormalities included the cessation of anther development, the arrest of pollen

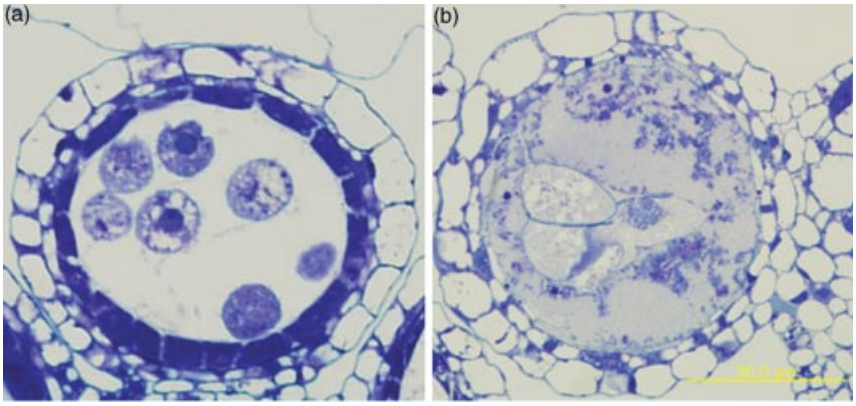

Figure 32.2 Cytological analysis of rice anther development (uninucleated stage) under cold stress. Rice plants were exposed to 16 °C, 10 h daylight. Cold stress resulted in abnormal swelling of tapetum layer and this abnormal swelling further dilated into the locular space, leading to abortion of microspores. (a) Unstressed (control). (b) Cold stressed.

development, anthers remaining within the flowers after anthesis, and partial or no dehiscence. Cytological observation revealed a dilation of tapetal layers in chilling-treated rice anthers [22, 24]. The dilation of tapetal layer was accompanied by a vigorous augmentation of cytoplasmic organelles such as mitochondria, proplastids, golgi bodies, and endoplasmic reticulum [22]. Chilling temperature treatment also affects the physiological status of anthers. Unpredictable cold snaps at the reproductive stage delay heading and results in pollen sterility, which was thought to be one of the key factors responsible for the reduction in grain yield of rice [24, 26–29]. The cytological observations of the tapetum under cold stress are shown in Figure 32.2.

32.3
Effect of Cold on Plant Physiology

Each plant has its unique set of temperature requirements that are optimum for its proper growth and development. A set of temperature conditions that are optimum for one plant may be stressful for another plant. Chilling-sensitive plants characteristically exhibit structural injuries and may suffer from metabolic dysfunction when chilled [30]. Chilling ultimately results in loss of membrane integrity, leading to solute leakage. Integrity of intracellular organelles is also disrupted, leading to the loss of compartmentalization and reduction and impairing of photosynthesis, protein assembly, and general metabolic processes. The physiological change in response to exposure to low temperature that triggers tolerance against freezing is known as "cold acclimation." The primary function of cold acclimation is to stabilize the integrity of cellular membranes against freezing-induced injury [31]. Cold acclimation also results in physical and biochemical restructuring of cell membranes through changes in the lipid composition, induction of other nonenzymatic proteins,

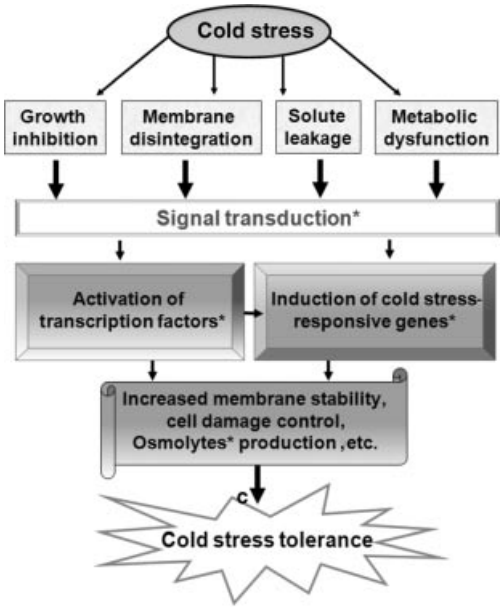

Figure 32.3 Cold stress response is perceived by plants through a signal transduction that leads to the activation of transcription factors and cold-responsive genes. Components involving in the rice cold stress tolerance are discussed in detail in the chapter (indicated by an asterisk in the figure).

enhancement of the antioxidative mechanisms, increased cellular sugar levels, and accumulation of cryoprotectants [32]. The stress response mechanism involves enzymes with biochemical roles that are believed to be major components of cellular defenses to protect cells against potential membrane damage [33]. These enzymes, such as Δ^1-pyrroline 5-carboxylate synthase, adjust the osmotic potential and thereby enhance the stability of cell membranes by increasing the desaturation of fatty acids in membrane lipids [34]. All these modifications help the plant to withstand and surpass the severe dehydration associated with cold stress. The responses of rice plants during cold stress and acclimation mechanisms are shown in Figure 32.3.

32.4
Transcription Factors in Rice Cold Stress

Understanding the mechanisms by which plants perceive environmental signals and transmit the signals to cellular machinery to activate adaptive responses is of fundamental importance to biology. Molecular studies have identified many genes that are induced or upregulated by stress [35–37]. Gene expression profiling using cDNA microarrays or gene chips has identified many more genes that are regulated by cold, drought, or salt stress [38–40]. Although the signaling pathways responsible

for the activation of these genes are largely unknown, transcriptional activation of some of the stress-responsive genes is understood to a great extent. In rice, numerous transcription factors have been found to play important roles in response to cold stress. The best characterized regulon of cold stress responses in plant contains transcription factor C-repeat binding factor/dehydration-responsive element binding factor (CBF/DREB) and its cold-inducible target genes, known as COR (cold-regulated gene), KIN (cold-induced gene), RD (responsive gene to dehydration), or LTI (low-temperature-induced gene) [41–49]. The promoters of RD29A (also known as COR78/LTI78) genes contain both ABRE and dehydration-responsive element (DRE)/CRT factors [50, 51]. Transcription factors belonging to the EREBP/AP2 family that bind to DRE/CRT were termed CBF1/DREB1B, CBF2/DREBC, and BF3/DREB1A [51, 54]. These transcription factor genes are induced early and transiently by cold stress and, in turn, activate the expression of target genes. Similar transcription factors DREB2A and DREB2B are activated by osmotic stress and may confer osmotic stress induction of target stress-responsive genes [53]. Several bZIP, bHLH, WRYK, MYB, NAC family members are involved in improving cold stress [55].

32.5
Improving Cold Stress Tolerance in Rice

Screening for genes involved in cold tolerance is an important initial step. Large numbers of studies are done on engineering cold stress tolerance in rice, essentially using compounds such as compatible solutes, membrane transporters, regulators of signal transduction or transcription, and cold-responsive genes.

For improving the cold stress tolerance in rice, any one or a combination of the following methods can be done:

1) Overexpression of transcription factors and cold stress-responsive genes
2) Increasing the production of osmolytes
3) Overexpression of cold stress signal transduction cascade genes

32.5.1
Overexpression of Transcription Factors and Cold Stress-Responsive Genes

Overexpression or enhanced expression of transcription factor could activate the genes involved in cold stress pathway. For example, overexpression of the transcription factor CBF1 leads to the induction of entire group of cold tolerance gene. All the cold tolerance-related genes contain a similar regulatory element in their promoters, the C-repeat (CRT) element/dehydration-responsive element/low-temperature-responsive element (LTRE). In this method, the transcription factor CBF1 directly binds to the CRT/DRE/LTRE elements in the promoter, and by activating transcription, it increases the expression of the corresponding genes involved in cold tolerance (Figure 32.4).

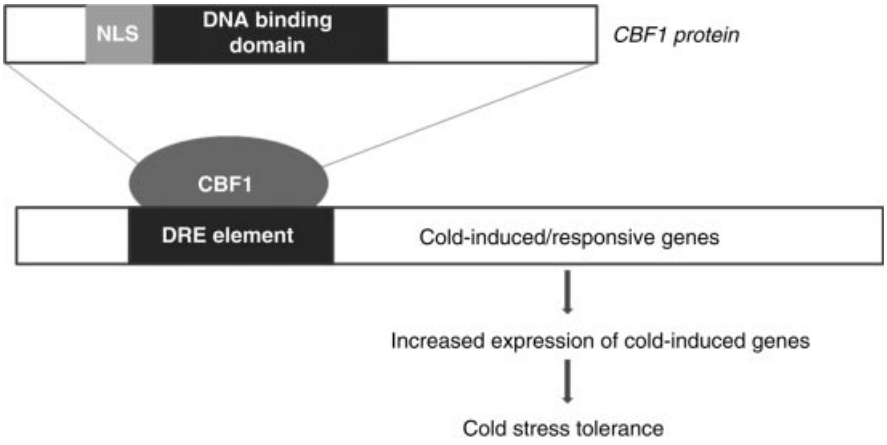

Figure 32.4 A number of cold-induced/responsive genes contain the DRE element in their promoters that is bound by transcription of the CBF family that activates the transcription. Overexpression of a single CBF gene induces the several cold-induced/responsive genes. NLS, nuclear localization signal.

Overexpression of zinc finger genes such as OsISAP8, OsCOIN, and OsISAP1 confers cold stress tolerance at the seedling stage [23, 56, 57]. Overexpression of OsbHLH1, OsDREB1/CBF, ROs-bZIP, SNAC2, and OsNAC6 also enhanced transgenic seedling resistance to chilling stress [49, 58–62]. Overexpression of OsMYB4 and OsMYB3R-2 significantly increased tolerance to freezing stress in transgenic *Arabidopsis* [63–65]. While in rice plants overexpression of OsMYB3R-2 led to higher transcript levels of several G2/M phase-specific genes, including OsCycB1;1, OsCycB2;1, OsCycB2;2, and OsCDC20.1, cold resistance mechanism in rice by OsMYB3R-2 could be mediated by regulating the cell cycle. Signaling components and metabolic regulators have also been shown to be involved in stress responses.

In rice, 33 aquaporins were reported by Sakurai *et al.* [66] and, more recently, 38 aquaporins by Forrest and Bhave [67]. Rice aquaporins are classified into four major families on the basis of their amino acid sequences: the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), Nod26-like intrinsic proteins (NIPs), and the small and basic intrinsic proteins (SIPs). These aquaporins when overexpressed in rice, it constitutively overexpressed in the leaf and root of transgenic plants, interacts with members of the subfamily, thereby improving the water balance of plants under low temperatures and resulting in the improved chilling tolerance of plants [68].

OsLti6 genes (OsLti6a and OsLti6b) encoding hydrophobic proteins homologous to *Arabidopsis* RCI2 enhanced tolerance to chilling stress in rice seedlings [46]. There are several reports regarding the enhanced tolerance of cold stress in rice. A list of transcription factors and genes improving the cold tolerance in rice is given in Table 32.1 and the rice genes showing tolerance in other plants are listed in Table 32.2.

Table 32.1 List of transcription factors and genes involved in enhancing the cold stress tolerance in rice.

Cultivar/variety	Mode of transformation	Construct	Mode of action	Reference
Nipponbare	Ag	p*35S-i(SODCC2)-codA-nos*t, p*35S-i (SODCC2)*-TP*(RBCS) -codA-nost*	Increase in glycine-betaine	[69]
Yamahoushi	Ag	p*UBI1-GPAT-nos*t	Increase in unsaturated fatty acids in phosphatidyl glycerol	[70]
Notohikari	Ag	p*35S-OSCDPK7-nos*t	Unknown posttranslational regulation mechanisms	[71]
Yamahoushi	Ag	*pUBI1-SGPAT-nost, pUBI-AGPAT-nost*	Increase in unsaturated fatty acids in phosphatidyl glycerol	[72]
Pusa Basmati 1	Ag	*pABA-TPSP-PINIIt, pRBCSTP (RBCS)-TPSP-PINIIt*	Trehalose accumulation	[73]
Sasnishiki	Ag	p*UBI-PSL029-nos*t	Detoxification of xenobiotic compounds and reactive oxygen species	[74]
Dongjin	Ag	*OsP5CS2*	Accumulation of proline	[75]
	Ag	p*35S-OSCDPK13-nost*	Signaling component	[76]
Nipponbare, Kita-ake	Ag	*OsDREB1A, OsDREB1B, AtDREB1A, AtDREB1B*, and *AtDREB1C*	Regulating the cold-responsive genes	[49]
Nipponbare, Kita-ake, Basmathi 370	Ag	OsCDPK13 and OsCRTintP1	Signaling components	[77]
Zhonghua 10	Ag	OsCOIN	Increasing the proline content	[56]
Kenjiandao10	Ag	AtICE1	Transcriptional activator	[78]
Nipponbare	Ag	p35S:OsDREB1F	Transcription factor	[79]
IR-50	Bombardment	pUBI-OsiSAP8-nost	Cytoplasmic zinc finger protein that acts in the signal transduction	[57]
Zhonghua 11	Ag	SNAC2	Transcription factor	[62]

Zhonghua 10	Ag	pUBI-OsMYB3R-2	Regulating the cell cycle	[65]
Zhonghua 11	Ag	p35S-ZFP245	Accumulation of proline, activation of the pyrroline-5-arboxylatesynthetase and proline transporter genes, and enhancement of the ROS-scavenging enzymes	[80]
Zhonghua 11	Ag	pUBI-OsPIP1;3-nost	Regulates the water balance of the plant	[68]
Dongjin	Ag	pUBI-OsAsr1-nost	Cold-responsive gene regulated by CBF1	[81]
Dongjin	Ag	pUBI-OsPRP3-nost	Increases the integrity of cell membrane and accumulation of free proline	[82]
Kita-ake	Ag	SICZFP1	Regulates cold-responsive genes	[83]
Tainung 67	Ag	pUBI-MYBS3	Activates cold signaling pathway	[84]
Nipponbare	Ag	p35S -TERF2	Increases accumulation of osmotic substances and chlorophyll, reduces ROS and malondialdehyde (MDA) content, and decreases electrolyte leakage	[85]
Zhongzuo 93	Ag	OVP1	Increased integrity of cell membrane, decreased MDA content, and accumulation of proline	[86]
		SNA	High levels of melatonin	[87]
		OsRAN2	Maintaining cell division through promoting the normal export of intranuclear tubulin at the end of mitosis	[88]

Table 32.2 List of rice genes conferring increased cold tolerance in other plants.

Genes	Plant	Mode of action	References
OsDREB1F	*Arabidopsis*	Transcription factor	[79]
OsiSAP8	Tobacco	Cytoplasmic zinc finger protein that is involved in the signal transduction	[57]
OsDREB1D	*Arabidopsis*	Transcription factor	[89]
OsSPX1	*Arabidopsis and tobacco*	Accumulation of proline and sugar	[90]
OsLTP	*Phalaenopsis amabilis*	Increased accumulation of total soluble sugars, proline, antioxidant superoxide dismutase	[91]
Osmyb4	*Osteospermum ecklonis, apple, Arabidopsis*	Transcription factor	[64, 92]
OrbHLH001	*Arabidopsis*	Involved in metabolic regulation or ionic homeostasis pathways in stress	[93]
SICZFP1	*Arabidopsis*	Regulating cold-responsive genes	[83]

32.5.2 Increasing the Production of Osmolytes

Particular interest has been focused on metabolites that can function as osmolytes. Osmolytes are involved in the regulation of cellular water relations and reduction of cellular dehydration. Their compatible solute behavior allows them to stabilize enzymes, membranes, and other cellular components. Osmolytes are also involved in retailoring of membrane lipid composition to optimize the liquid/crystalline physical structure necessary for proper membrane function and energy sources. Such stress-responsive metabolites particularly include soluble sugars, amino acids, organic acids, polyamines, and lipids [10, 94]. Plants experience cold or chilling stress at temperatures from 0 to 15 °C. Under such situations, plants try to maintain homeostasis to acquire freezing tolerance and this involves extensive reprogramming of gene expression and metabolism [4, 95].

A wide range of compatible solutes are produced by plants. These compounds fall into two broad classes: (i) sugars and sugar alcohol and (ii) zwitterionic compounds. The first class includes sugar alcohols such as mannitol, sorbitol, pinitol, and D-ononitol and oligosaccharides such as trehalose and fructans. The second class includes amino acids such as proline and quaternary ammonium compounds such as glycine-betaine. Overproduction of various compatible solutes has been tested in rice, for example, glycine-betaine, trehalose, and proline, to achieve significant cold tolerance. A list of compounds improving rice cold tolerance is given in Table 32.3.

Table 32.3 List of compatible solutes conferring cold tolerance in rice.

Compatible solutes	Mode of action	References
Glycine-betaine	Increase in glycine-betaine	[69, 96–98]
Trehalose	Trehalose accumulation and activation of stress-responsive genes	[73, 99–101]
Proline	Accumulation of free proline	[75, 82]

A few osmolytes are discussed here; for example, OsTPP1, a gene encoding a trehalose-6P phosphatase in rice, when overexpressed in rice confers cold stress tolerance by trehalose accumulation and by activation of stress-responsive genes.

Free proline is known to be one of the compatible osmolytes preventing dehydration in response to freezing and drought stress. Increase in proline content occurs in many plant species during cold acclimation. Proline is also known to protect membranes and proteins against the adverse effects of temperature extremes. Rapid catabolism of proline upon relief of stress may provide reducing equivalents that support mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress-induced damage. Our recent report indicated that overexpression of proline-rich protein OsPRP3, which increases the free proline content in the transgenic plants, leads to the enhancement of the cell wall integrity in the cold-tolerant plant and confers cold tolerance in rice [82]. Moreover, proline-rich protein/glycoproteins are thought to play an integral role in extracellular matrix structure of many plant cells by adding mechanical strength to the cell wall and assisting in proper wall assembly.

32.5.3
Overexpression of Cold Stress Signal Transduction Cascade Genes

Plants are continuously exposed to various environmental stimuli during their life cycle. To maintain their life from such stresses, several mechanisms for sensing stimuli and activating immune responses are evolutionarily conserved. Particularly, MAPK cascades are well known as a universal signal module consisting of a MAPK kinase kinase (MAPKKK) that mediates the phosphorylation of a MAPK kinase that in turn phosphorylates a MAPK. The MAPK phosphorylation system serves as a link in various ways between upstream receptors and downstream targets and thereby controls many important cellular functions [102, 103]. MAPKs participate in signal perception and transfer and then induce rapidly and correctly necessary information for adaptation from different stimuli including cold stress. For example, in rice, two components of an MAPK pathway, OsMEK1 and OsMAP1, are induced by low temperature and involved in low-temperature stress [104], and also overexpression of OsMAPK5 conferred tolerance to cold stress in rice seedlings.

In addition to MAPKs, in rice CDPKs (CaM domain-containing protein kinases) are found to be upregulated by cold. Stress-responsive CIPK genes encoding

calcineurin B-like protein interacting protein kinases such as OsCIPK03, OsCIPK12, and OsCIPK15 also play important roles in improving the tolerance to chilling stress in rice [105]. The comprehensive analysis of CDPKs in rice shows that OsCPK4, OsCPK5, and OsCPK13 (OsCDPK7) are upregulated in response to cold [106]. The overexpression of OsCDPK7 in rice resulted in improvement of cold, salt, and drought stress tolerance [107]. In another study, the overexpression of OsCDPK13 and calreticulin interacting protein (CRTintP1) conferred cold tolerance to rice [77].

32.6 Conclusions and Perspective

Cold stress signaling is an important area with respect to increasing the productivity in rice. Therefore, the basic understanding of the mechanisms underlying the functioning of stress genes is important for the development of transgenic plants. Each stress is a multigenic trait, and therefore their manipulation may result in alteration of a large number of genes as well as their products. A deeper understanding of the transcription factors regulating these genes, the products of the major stress-responsive genes, and crosstalk between different signaling components should remain an area of intense research activity in future. The knowledge generated through these studies should be utilized in making transgenic plants that would be able to tolerate stress condition. In the improvement of crops, it is very important to perturb the natural machinery as minimum as possible and activate the stress genes at the correct time. Therefore, it is desirable that appropriate stress-inducible promoters should drive both stress genes and transcription factors, which will minimize their expression under a nonstressed condition, thereby reducing yield penalty. The product of these genes should also be targeted to the desired tissue as well as cellular location to control both timing and intensity of expression.

References

1 Fitter, A.H. and Hay, R.K.M. (1981) *Environmental Physiology of Plants*, Academic Press, New York.

2 Kotak, S., Larkindale, J., Lee, U., von Koskull-Doring, P., Vierling, E., and Scharf, K.D. (2007) Complexity of the heat stress response in plants. *Curr. Opin. Plant Biol.*, **10**, 310–316.

3 Somerville, C. (1995) Direct tests of the role of membrane lipid composition in low temperature-induced photoinhibition and chilling sensitivity in plants and cyanobacteria. *Proc. Natl Acad. Sci. USA*, **92**, 6215–6218.

4 Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 571–599.

5 Larkindale, J., Hall, J.D., Knight, M.R., and Vierling, E. (2005) Heat stress phenotypes of *Arabidopsis* mutants implicate multiple signaling pathways in the acquisition of thermotolerance. *Plant Physiol.*, **138**, 882–888.

6 Zhu, J., Dong, C.H., and Zhu, J.K. (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr. Opin. Plant Biol.*, **10**, 290–295.

7 Kubien, D.S., von Caemmerer, S., Furbank, R.T., and Sage, R.F. (2003) C4 photosynthesis at low temperature. A study using transgenic plants with reduced amounts of Rubisco. *Plant Physiol.*, **132**, 1577–1585.

8 Schwender, J., Ohlrogge, J., and Shachar-Hill, Y. (2004) Understanding flux in plant metabolic networks. *Curr. Opin. Plant Biol.*, **7**, 309–317.

9 Fernie, A.R., Geigenberger, P., and Stitt, M. (2005) Flux an important, but neglected, component of functional genomics. *Curr. Opin. Plant Biol.*, **8**, 174–182.

10 Nayyar, H., Chander, K., Kumar, S., and Bains, T. (2005) Glycine betaine mitigates cold stress damage in chickpea. *Agron. Sustain. Dev.*, **25**, 381–388.

11 Cruz, R.P. and Milach, S.C.K. (2004) Cold tolerance at the germination stage of rice: methods of evaluation and characterization of genotypes. *Sci. Agric.*, **61**, 1–8.

12 Chuong, P.V. and Omura, T. (1982) Studies on the chlorosis expressed under low temperature in rice, *Oryza sativa* L., in *Bulletin of the Institute of Tropical Agriculture*, vol. **5**, Kyushu University, Fukuoka, pp. 1–58.

13 Kwak, T.S., Vergara, B.S., Nanda, J.S., and Coffman, W.R. (1984) Inheritance of seedling cold tolerance in rice. *SABRAO J.*, **16**, 83–86.

14 Nagamine, T. (1991) Genic control of tolerance to chilling injury at seedling stage in rice. *Jpn. J. Breed.*, **41**, 35–40.

15 Andaya, V.C. and Mackill, D.J. (2003) Mapping of QTLs associated with cold tolerance during the vegetative stage in rice. *J. Exp. Bot.*, **54**, 2579–2585.

16 Fujino, K., *et al.* (2004) Mapping of quantitative loci controlling low temperature germinability in rice (*Oryza sativa*). *Theor. Appl. Genet*, **108**, 794–799.

17 Lou, Q., Chen, L., Sun, Z., Xing, Y., Li, J., Xu, X., *et al.* (2007) A major QTL associated with cold tolerance at seedling stage in rice (*Oryza sativa* L.). *Euphytica*, **158**, 87–94.

18 Misawa, S., *et al.* (2000) Mapping of QTLs for low temperature response in seedlings of rice (*Oryza sativa* L.). *Cereal Res. Commun.*, **28**, 33–40.

19 Miura, K., Lin, S.Y., Yano, M., and Nagamine, T. (2001) Mapping quantitative trait loci controlling low-temperature germinability in rice (*Oryza sativa* L.). *Breed. Sci.*, **51**, 293–299.

20 Zhang, Z.H., Su, L., Li, W., Chen, W., and Zhu, Y.G. (2004) A major QTL conferring cold tolerance at the early seedling stage using recombinant inbred lines of rice (*Oryza sativa* L.). *Plant Sci.*, **168**, 527–534.

21 Suh, J.P., *et al.* (2010) Identification and analysis of QTLs controlling cold tolerance at the reproductive stage and validation of effective QTLs in cold-tolerant genotypes of rice (*Oryza sativa* L.). *Theor. Appl. Genet.*, **120**, 985–95.

22 Nishiyama, I. (1984) Climatic influence on pollen formation and fertilization, in *Biology of Rice* (eds S. Tsunoda and N. Takahashi), Japan Scientific Societies Press, Tokyo, pp. 153–171.

23 Mukhopadhyay, A., Vij, S., and Tyagi, A.K. (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc. Natl. Acad. Sci. USA*, **101**, 6309–6314.

24 Gothandam, K.M., Kim, E.S., and Chung, Y.Y. (2007) Ultrastructural study of rice tapetum under low temperature stress. *J. Plant Biol.*, **50**, 396–402.

25 Satake, T. and Hayase, H. (1970) Male sterility caused by cooling treatment at the young microspore stage in rice plants. V. Estimations of pollen developmental stage and the most sensitive stage to coolness. *Proc. Crop Sci. Soc. Jpn.*, **39**, 468–473.

26 Kaneda, C.B.H. (1974) Response of indica-japonica rice hybrids to low temperatures. *SABRAO J.*, **6**, 17–32.

27 Mackill, D.J.L.X. (1997) Genetic variation for traits related to temperate adaptation of rice cultivars. *Crop Sci.*, **37**, 1340–1346.

28 Andaya, V.C. and Tai, T.H. (2006) Fine mapping of the qCTS12 locus, a major QTL for seedling cold tolerance in rice. *Theor. Appl. Genet.*, **113**, 467–475.

29 Suzuki, K., Nagasuga, K., and Okada, M. (2008) The chilling injury induced by high root temperature in the leaves of rice seedlings. *Plant Cell Physiol.*, **49**, 433–442.

30 Kacperska, A. (1999) Plant responses to low temperature: signaling pathways involved in plant acclimation, in *Cold-adapted Organisms: Ecology, Physiology, Enzymology and Molecular Biology* (eds R. Margesin and F. Schinner), Springer, Berlin, pp. 79–103.

31 Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.*, **444**, 139–158.

32 Xin, Z. and Browse, J. (2000) Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant Cell Environ.*, **23**, 893–902.

33 Fowler, S. and Thomashow, M. (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell*, **14**, 1675–1690.

34 Steponkus, P., Uemura, M., Joseph, R., Gilmour, S., and Thomashow, M. (1998) Mode of action of the COR15a gene on the freezing tolerance of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, **95**, 14570–14575.

35 Ingram, J. and Bartel, D. (1996) The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 377–403.

36 Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.*, **2**, 48–54.

37 Zhu, J.K., Hasegawa, P.M., and Bressan, R.A. (1997) Molecular aspects of osmotic stress in plants. *CRC Crit. Rev. Plant Sci.*, **16**, 253–277.

38 Bohnert, H.J., *et al.* (2001) A genomics approach towards salt stress tolerance. *Plant Physiol. Biochem.*, **39**, 295–311.

39 Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., and Bohnert, H. (2001) Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell*, **13**, 889–905.

40 Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninic, P., Hayashizaki, Y., and Shinozaki, K. (2001) Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell*, **13**, 61–72.

41 Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994) The 5′-region of *Arabidopsis thaliana* cor15a has *cis*-acting elements that confer cold-, drought-and ABA-regulated gene expression. *Plant Mol. Biol.*, **24**, 701–713.

42 Dubouzet, J.G., *et al.* (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought, high salt and cold-responsive gene expression. *Plant J.*, **33**, 751–763.

43 Rabbani, M.A., *et al.* (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant physiol.*, **133**, 1755.

44 Beer, M.A. and Tavazoie, S. (2004) Predicting gene expression from sequence. *Cell*, **117**, 185–198.

45 Yazaki, J., *et al.* (2004) Transcriptional profiling of genes responsive to abscisic acid and gibberellin in rice: phenotyping and comparative analysis between rice and *Arabidopsis*. *Physiol. Genomics*, **17**, 87–100.

46 Morsy, M.R., Almutairi, A.M., Gibbons, J., Yun, S.J., and de Los Reyes, B.G. (2005) The OsLti6 genes encoding low-molecular-weight membrane proteins are differentially expressed in rice cultivars with contrasting sensitivity to low temperature. *Gene*, **344**, 171–180.

47 Agarwal, M., *et al.* (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J. Biol. Chem.*, **281**, 37636.

48 Benedict, C., Geisler, M., Trygg, J., Huner, N., and Hurry, V. (2006) Consensus by democracy. Using meta-analyses of microarray and genomic data to model the cold acclimation signaling pathway in *Arabidopsis*. *Plant Physiol.*, **141**, 1219.

49 Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol.*, **47**, 141–153.

50 Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell*, **6**, 251–264.

51 Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA*, **94**, 1035–1040.

52 Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M., and Thomashow, M.F. (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *Plant J.*, **16**, 433–442.

53 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*, **10**, 1391–1406.

54 Medina, J., Bargues, M., Terol, J., Perez-Alonso, M., and Salinas, J. (1999) The *Arabidopsis* CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. *Plant Physiol.*, **119**, 463–470.

55 Yun, K.Y., *et al.* (2010) Transcriptional regulatory network triggered by oxidative signals configures the early response mechanisms of japonica rice to chilling stress. *BMC Plant Biol.*, **10**, 16.

56 Liu, K., Wang, L., Xu, Y., Chen, N., Ma, Q., Li, F., and Chong, K. (2007) Overexpression of OsCOIN, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice. *Planta*, **226**, 1007–1016.

57 Kanneganti, V. and Gupta, A.K. (2008) Overexpression of OsiSAP8, a member of stress associated protein (SAP) gene family of rice confers tolerance to salt, drought and cold stress in transgenic tobacco and rice. *Plant Mol. Biol.*, **66**, 445–462.

58 Wang, Y.J., Zhang, Z.G., He, X.J., Zhou, H.L., Wen, Y.X., Dai, J.X., Zhang, J.S., and Chen, S.Y. (2003) A rice transcription factor OsbHLH1 is involved in cold stress response. *Theor. Appl. Genet.*, **107**, 1402–1409.

59 Ohnishi, T., Sugahara, S., Yamada, T., Kikuchi, K., Yoshiba, Y., Hirano, H.Y., and Tsutsumi, N. (2005) OsNAC6, a member of the NAC gene family, is induced by various stresses in rice. *Genes Genet. Syst.*, **80**, 135–139.

60 Cheng, C., Yun, K.Y., Ressom, H.W., Mohanty, B., Bajic, V.B., Jia, Y., Yun, S.J., and de Los Reyes, B.G. (2007) An early response regulatory cluster induced by low temperature and hydrogen peroxide in seedlings of chilling-tolerant japonica rice. *BMC Genomics*, **8**, 175.

61 Nakashima, K., Tran, L.S.P., Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., Ito, Y., Hayashi, N., Shinozaki, K., and Yamguchi-Shinozaki, K. (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. *Plant J.*, **51**, 617–630.

62 Hu, H., You, J., Fang, Y., Zhu, X., Qi, Z., and Xiong, L. (2008) Characterization of

transcription factor gene SNAC2 conferring cold and salt tolerance in rice. *Plant Mol. Biol.*, **67**, 169–181.

63 Vannini, C., Locatelli, F., Bracale, M., Magnani, E., Marsoni, M., Osnato, M., Mattana, M., Baldoni, E., and Coraggio, I. (2004) Overexpression of the rice *Osmyb4* gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. *Plant J.*, **37**, 115–127.

64 Pasquali, G., Biricolti, S., Locatelli, F., Baldoni, E., and Mattana, M. (2008) Osmyb4 expression improves adaptive responses to drought and cold stress in transgenic apples. *Plant Cell Rep.*, **27**, 1677–1686.

65 Ma, Q., *et al.* (2009) Enhanced tolerance to chilling stress in OsMYB3R-2 transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. *Plant Physiol.*, **150**, 244–256.

66 Sakurai, J., Ishikawa, F., Yamaguchi, T., Uemura, M., and Maeshima, M. (2005) Identification of 33 rice aquaporin genes and analysis of their expression and function. *Plant Cell Physiol.*, **46**, 1568–1577.

67 Forrest, K.L. and Bhave, M. (2007) Major intrinsic proteins (MIPs) in plants: a complex gene family with major impacts on plant phenotype. *Funct. Integr. Genomics*, **7**, 263–289.

68 Matsumoto, T., Lian, H.L., Su, W.A., Tanaka, D., Liu, C.W., Iwasaki, I., and Kitagawa, Y. (2009) Role of the aquaporin PIP1 subfamily in the chilling tolerance of rice. *Plant Cell Physiol.*, **50**, 216–229.

69 Sakamoto, A., Alia, and Murata, N. (1998) Metabolic engineering of rice leading to biosynthesis of glycine betaine and tolerance to salt and cold. *Plant Mol. Biol.*, **38**, 1011–1019.

70 Yokoi, S., Higashi, S., Kishitani, S., Murata, N., and Toriyama, K. (1998) Introduction of the cDNA for *Arabidopsis* glycerol-3-phosphate acyltransferase (*GPAT*) confers unsaturation of fatty acids and chilling tolerance of photosynthesis on rice. *Mol. Breed.*, **4**, 269–275.

71 Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., and Izui, K. (2000) Overexpression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J.*, **23**, 319–327.

72 Ariizumi, T., Kishitani, S., Inatsugi, R., Nishida, I., Murata, N., and Toriyama, K. (2002) An increase in unsaturation of fatty acids in phosphatidylglycerol from leaves improves the rates of photosynthesis and growth at low temperatures in transgenic rice seedlings. *Plant Cell Physiol.*, **43**, 751–758.

73 Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V., and Wu, R.J. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc. Natl. Acad. Sci. USA*, **99**, 15898–15903.

74 Takesawa, T., Ito, M., Kanzaki, H., Kameya, N., and Nakamura, I. (2002) Overexpression of glutathione *S*-transferase in transgenic rice enhances germination and growth at low temperature. *Mol. Breed.*, **9**, 93–101.

75 Hur, J., Jung, K.H., Lee, C.H., and An, G. (2004) Stress-inducible *OsP5CS2* gene is essential for salt and cold tolerance in rice. *Plant Sci.*, **167**, 417–426.

76 Abbasi, F., Onodera, H., Toki, S., Tanaka, H., and Komatsu, S. (2004) *OsCDPK13*, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. *Plant Mol. Biol.*, **55**, 541–552.

77 Komatsu, S., Yang, G., Khan, M., Onodera, H., Toki, S., and Yamaguchi, M. (2007) Overexpression of calcium-dependent protein kinase 13 and calreticulin interacting protein 1 confers cold tolerance on rice plants. *Mol. Genet. Genomics*, **277**, 713–723.

78 Xiang, D.-J., *et al.* (2008) Over-expression of ICE1 Gene in transgenic rice improves cold tolerance. *Rice Sci.*, **15**, 173–178.

79 Wang, Q., Guan, Y., Wu, Y., Chen, H., Chen, F., and Chu, C. (2008) Overexpression of a rice OsDREB1F gene increases salt, drought, and low temperature tolerance in both *Arabidopsis* and rice. *Plant Mol. Biol.*, **67**, 589–602.

80 Huang, J., Sun, S.-J., Xu, D.-Q., Yang, X., Bao, Y.-M., Wang, Z.-F., Tang, H.-J., and Zhang, H. (2009) Increased tolerance of rice to cold, drought and oxidative stresses mediated by the overexpression of a gene that encodes the zinc finger protein ZFP245. *Biochem. Biophys. Res. Commun.*, **389**, 556–561.

81 Kim, S.-J., Lee, S.-C., Hong, S.K., An, K., An, G., and Kim, S.-R. (2009) Ectopic expression of a cold-responsive OsAsr1 cDNA gives enhanced cold tolerance in transgenic rice plants. *Mol. Cells*, **27** (4), 449–458.

82 Gothandam, K.M., Nalini, E., Karthikeyan, S., and Shin, J.S. (2010) OsPRP3, a flower specific proline-rich protein of rice, determines extracellular matrix structure of floral organs and its overexpression confers cold-tolerance. *Plant Mol. Biol.*, **72**, 125–135.

83 Zhang, X., *et al.* (2010) Overexpression of SlCZFP1, a novel TFIIIA-type zinc finger protein from tomato, confers enhanced cold tolerance in transgenic *Arabidopsis* and rice. *Plant Mol. Biol. Rep.*, 1–12.

84 Su, C.F., *et al.* (2010) A novel MYBS3-dependent pathway confers cold tolerance in rice. *Plant Physiol.*, **153** (1), 145–158.

85 Tian, Y., *et al.* (2010) Overexpression of ethylene response factor TERF2 confers cold tolerance in rice seedlings. *Transgenic Res.* **20** (4), 857–866.

86 Zhang, J., Li, J., Wang, X., and Chen, J. (2011) OVP1, a vacuolar H^+-translocating inorganic pyrophosphatase (V-PPase), overexpression improved rice cold tolerance. *Plant Physiol. Biochem.*, **49**, 33–38.

87 Kang, K., *et al.* (2010) Enhanced production of melatonin by ectopic overexpression of human serotonin *N*-acetyltransferase plays a role in cold resistance in transgenic rice seedlings. *J. Pineal Res*, **49**, 176–182.

88 Chen, N., *et al.* (2011) *OsRAN2*, essential for mitosis, enhances cold tolerance in rice by promoting export of intranuclear tubulin and maintaining cell division under cold stress. *Plant Cell Environ*, **34**, 52–64.

89 Zhang, Y., Chen, C., Jin, X.-F., Xiong, A.-S., Peng, R.-H., Hong, Y.-H., Yao, Q.-H., and Chen, J.-M. (2009) Expression of a rice DREB1 gene, OsDREB1D, enhances cold and high-salt tolerance in transgenic *Arabidopsis*. *BMB Rep.*, **42** (8), 486–492.

90 Zhao, L., Liu, F., Xu, W., Di, C., Zhou, S., Xue, Y., Yu, J., and Su, Z. (2009) Increased expression of OsSPX1 enhances cold/subfreezing tolerance in tobacco and *Arabidopsis thaliana*. *Plant Biotechnol. J.*, **7** (6), 550–561.

91 Qin, X., Liu, Y., Mao, S., Li, T., Wu, H., Chu, C., and Wang, Y. (2011) Genetic transformation of lipid transfer protein encoding gene in *Phalaenopsis amabilis* to enhance cold resistance. *Euphytica*, **177** (1), 33–43.

92 Laura, M., *et al.* (2010) Metabolic response to cold and freezing of *Osteospermum ecklonis* overexpressing Osmyb4. *Plant Physiol. Biochem*, **48** (9), 764–771.

93 Li, F., Guo, S., Zhao, Y., Chen, D., Chong, K., and Xu, Y. (2010) Overexpression of a homopeptide repeat-containing bHLH protein gene (OrbHLH001) from Dongxiang wild rice confers freezing and salt tolerance in transgenic *Arabidopsis*. *Plant Cell Rep.*, **29** (9), 977–986.

94 Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., and Basra, S.M.A. (2009) Plant drought stress: effects, mechanisms and management. *Agron. Sustain. Dev.*, **29**, 185–212.

95 Cook, D., Fowler, S., Fiehn, O., and Thomashow, M.F. (2004) A prominent role for the CBF cold response pathway in configuring the low temperature metabolome of *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **101**, 15243–15248.

96 Mohanty, A., Kathuria, H., Ferjani, A., Sakamoto, A., Mohanty, P., Murata, N., and Tyagi, A.K. (2002) Transgenic plants of an elite *indica* rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. *Theor. Appl. Genet.*, **106**, 51–57.

97 Sawahel, W. (2003) Improved performance of transgenic glycine betaine accumulating rice plants

under drought stress. *Bol. Plant.*, **47**, 39–44.

98 Su, J., Hirji, R., Zhang, L., He, C., Selvaraj, G., and Wu, R. (2006) Evaluation of the stress-inducible production of choline oxidase in transgenic rice as a strategy for producing the stress-protectant glycine betaine. *J. Exp. Bot.*, **57**, 1129–1135.

99 Jang, M.H., *et al.* (2003) Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. *Plant Physiol.*, **131**, 516–524.

100 Pramanik, M.H. and Imai, R. (2005) Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. *Plant Mol. Biol.*, **58**, 751–762.

101 Ge, L.F., Chao, D.Y., Shi, M., Zhu, M.Z., Gao, J.P., and Lin, H.X. (2008) Overexpression of the trehalose-6-phosphate phosphatase gene OsTPP1 confers stress tolerance in rice and results in the activation of stress responsive genes. *Planta*, **228**, 191–201.

102 Nakagami, H., Pitzschke, A., and Hirt, H. (2005) Emerging MAP kinase pathways in plant stress signalling. *Trends Plant Sci.*, **10** (7), 339–346.

103 Mishra, N.S., Tuteja, R., and Tuteja, N. (2006) Signaling through MAP kinase networks in plants. *Arch. Biochem. Biophys.*, **452** (1), 55–68.

104 Wen, J.Q., Oono, K., and Imai, R. (2002) The mitogen-activated protein MAP kinase cascade pathway is among the most well-characterized signal transduction systems in animals, yeast, and plants. *Plant Physiol.*, **129**, 1880–1891.

105 Xiang, Y., Huang, Y., and Xiong, L. (2007) Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. *Plant Physiol.*, **144**, 1416–1428.

106 Ray, S., Agarwal, P., Arora, R., Kapoor, S., and Tyagi, A.K. (2007) Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (*Oryza sativa* L. ssp. *indica*). *Mol. Genet. Genomics*, **278** (5), 493–505.

107 Saijo, Y., Kinoshita, N., Ishiyama, K., Hata, S., Kyozuka, J., Hayakawa, T., Nakamura, T., Shimamoto, K., Yamaya, T., and Izui, K. (2001) A Ca^{2+}-dependent protein kinase that endows rice plants with cold- and salt-stress tolerance functions in vascular bundles. *Plant Cell Physiol.*, **42**, 1228–1233.

33
Maize: Physiological and Molecular Approaches for Improving Drought Tolerance

Ishwar Singh, Thirunavukkarasu Nepolean, Rajyalakshmi Ambika Rajendran, and Mariko Shono

Maize is a C_4 crop with a high rate of photosynthetic activity, leading to high grain and biomass yield. It is predominantly a cross-pollinating species, a feature that has contributed to its broad genetic and morphological variability and geographical adaptability. Economically, the most important types of maize are grown for grain and fodder or silage production. However, in the tropics, grain is primarily used for human consumption. FAO predicts that an additional 60 Mt of maize grain will be needed from the annual global harvest by 2030. The demand for maize as an animal feed will continue to grow faster than the demand for its use as a human food, particularly in Asia, where a doubling of production is expected from the present level of 165 Mt to almost 400 Mt in 2030.

33.1
Introduction

One of the focal points of global food security is the ability to produce the crop all year round in variable climatic conditions, including unpredictable rainfall, low soil moisture, excess soil moisture, excess heat, and so on. Among all, drought poses a major hindrance to this objective in many tropical countries [1]. The ability of plants to tolerate moisture stress condition is crucial for sustaining the agricultural production worldwide. Recent studies of molecular and genomic experiments have increased the understanding of the regulatory and functional networks controlling the drought stress response and have led to practical approaches for developing drought tolerance in plants [2].

From an application point of view, it is important to select genotypes that are able to optimize water harvest and use water efficiently, while maximizing yield in relation to the dynamics of the drought episodes prevailing in each target environment. The objective of this review is to consolidate the current emerging trends of physiology, molecular breeding, and functional genomics that would be influential in integrating breeding and genetic engineering approaches for development of drought-tolerant genotypes in maize.

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33.2
Basic Concept of Drought Tolerance and its Significance

Drought is a meteorological term and can be defined as the absence of adequate moisture necessary for normal plant growth and to complete the life cycle. Drought in agriculture is due to shortage of water in the root zone, resulting in yield reduction, which is the principal concern of this chapter. The lack of adequate moisture leading to water stress is a common occurrence in rain-fed areas, brought about by infrequent rains as well as inadequate and poor irrigation [3]. Growth and other processes are progressively retarded as soil water content decreases below field capacity. However, most of the plant physiological processes are influenced directly by plant water stress and only indirectly by soil and atmospheric water stress. As much as 17–60% loss in maize yield could be recorded per year in tropical countries due to moisture stress. Hence, the conservation of the soil moisture is essential in the dry season of the crop growth [4].

The term drought tolerance relates to ultimate yield rather than to the capacity of the plant to survive in water-limited conditions [5]. Tolerance consists of drought avoidance and dehydration tolerance that are ultimately measured by the reproductive success of the species [6]. Those plants able to reproduce are represented in the next generation. For grain crops, the measure is similar but determined as yield per unit area of land. Drought avoidance strategies in plants include deep rooting traits, conservative use of available water to ensure that the grain filling is completed, crop life cycle to match rainfall, and short-duration genotypes to escape from the drought. Dehydration tolerance involves plants' ability to partially dehydrate but remain viable and resume growth when water is available.

33.3
Impact of Drought on Phonological Phases of Maize

Moisture stress particularly affects the ability of the maize plant to produce grain at three critical stages of growth: early in the growing season (seedling emergence), at flowering, and during mid-to-late grain filling. Moisture stress during flowering and pollination leads to pollen and silk sterility, inadequate partitioning of source, and improper mobilization from source to sink, which in turn cause maximum yield penalty (Figure 33.1). In fact, silking or the onset of the reproductive stage is the most sensitive stage and will result in 100% yield loss when moisture stress is accompanied with heat stress [7]. In India, moisture stress, particularly at reproductive stage, has been identified as the most important limiting factor of maize production and productivity.

In tropics, moisture stress at the beginning of a season can damage plant stands; however, crop can revive when it gets water. On the other hand, grain yield reductions from mid-to-late grain filling are not nearly as severe as those produced by a similar stress during flowering.

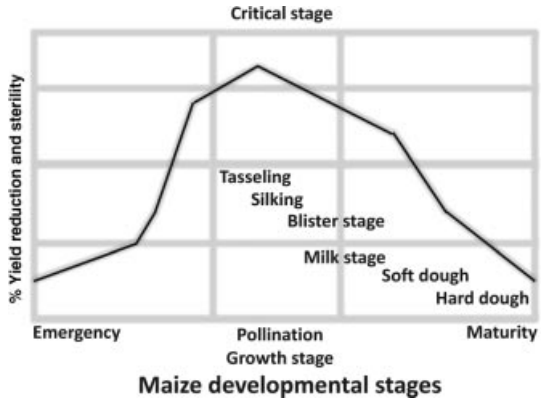

Figure 33.1 Impact of drought stress at different stages of maize development [8]. Reprinted from [8] with kind permission from ASA, CSSA, and SSSA Book Publishing.

Reduction of plant water status to the wilting point during the preflowering, flowering, and postflowering stages results in yield reduction by 25, 50, and 21%, respectively [9]. Silk growth, kernel size, and number appear to depend directly on the flow of photosynthates produced during the 3 weeks of extreme sensitivity period that bracket flowering [10]. Drought also lessens the capacity of developing kernels to use available assimilates because of the impaired functioning of a key enzyme acid invertase [11, 12]. Once kernels enter the linear phase of biomass accumulation about 2–3 weeks after pollination, they develop the capacity to access reserve assimilates stored in the stem and husk. If kernels successfully reach this stage, they will normally grow to at least 30% of the weight of kernels on unstressed plants, even in the presence of severe moisture stress [13]. Plants also respond and adapt to water deficit at both cellular and molecular levels, for instance, by accumulation of osmolytes and proteins specifically involved in stress tolerance. The physiological mechanisms involved in cellular and whole-plant responses to water stress, therefore, generate considerable interest.

33.3.1
Physiological, Morphological, and Metabolic Changes Induced by Drought

During drought, certain morphological, physiological, and metabolic changes occur in response to drought that allow the plant to avoid water loss by continuous water uptake at reduced water potential or to tolerate a reduced tissue water content [14]. Numerous physiological and biochemical changes occur in response to drought stress in various plant species. Changes in protein expression, accumulation, and synthesis have been observed in many plant species as a result of plant exposure to drought stress during growth. The physiological responses of plants to a deficit of water include leaf wilting, a reduction in leaf area, leaf abscission, and the stimulation of root growth by directing nutrients to the underground parts of the plants.

Plant cells are required to maintain water balance. To maintain this water balance, plants absorb water when water potential is negative. Cells can decrease their water potential through the accumulation of solutes, such as sugars, amino acids, organic acids, and ions, especially potassium (K^+). As cellular enzymes are severely inhibited by the presence of ions, these must be removed from the cytosol (the ground fluid substance of the cell) and stored in special storage cell organelles, the vacuoles. Plants resort to many adaptive strategies in response to abiotic environmental stresses such as dehydration and excessive osmotic pressure. These adaptive mechanisms include changes in physiological, morphological, and biochemical processes.

33.3.2 Physiological Changes

Physiological changes at the cellular level associated with drought stress include accumulation of osmolytes, turgor loss, reduction in photosynthetic activity, and changes in membrane fluidity. Abscisic acid (ABA) production is also induced and leads to a further loss of stomatal turgor. The resulting stomatal closure causes a concomitant decrease in CO_2 availability in the leaves, and hence in assimilates availability to the plant [15].

During moisture stress, the xylem vessels give up contents such as ABA to the leaf apoplast, thereby increasing the hormone concentration in this compartment. ABA is carried with the transpiration stream inside the leaf around and through the mesophyll cells so that it reaches the stomatal guard cells of the epidermis that contain ABA receptors with external (and possibly internal) loci in their plasma membranes. Once bound, the hormone induces an internal signal transduction cascade, usually involving increase in both externally and internally sourced cytoplasmic calcium, which eventually reduces the osmotic potential of guard cells via loss of K^+ and Cl^- with stomatal closure as a consequence [16].

Although the photosynthetic machinery has a range of photoprotective mechanisms to dissipate excess light energy, the continued exposure of leaves to excessive excitation energy can lead to photoreduction of oxygen and the generation of highly toxic reactive oxygen species (ROS), such as superoxides and peroxides. These free radicals are harmful compounds causing chemical damage to DNA and proteins and can therefore have lethal effects on cellular metabolism [17]. Plants have evolved several strategies to deal with ROS, including the production of chemical antioxidants such as ascorbic acid, glutathione, and α-tocopherol that directly remove potentially damaging electrons from the ROS, and also peroxidases and superoxide dismutases that scavenge the electrons enzymatically [18].

Another adaptive mechanism for protection against drought is the maintenance of turgor during periods of drought by adjusting the osmotic pressure of cells. There are two main routes whereby this can be achieved. First, the cell can sequester ions into cellular compartments. Second, specialized osmolytes such as proline, glycine-betaine, mannitol, trehalose, ononitol, and ectoine can be synthesized to readjust cellular osmotic potential. These osmolytes are also active in scavenging ROS, especially if they are targeted to the chloroplast [19]. Other specialized organic

molecules can be used to protect cellular membranes against physical damage and proteins against unfolding. Dehydration induces the partitioning of amphiphilic molecules such as glycosylated flavonols and hydroquinones into membranes; these compounds increase membrane fluidity and depress phase transition temperatures [15]. During extreme desiccation, tolerant plants synthesize large amounts of nonreducing disaccharides, such as trehalose, which can substitute for water by satisfying hydrogen bonding requirements of polar amino acid residues at protein surfaces and maintain the folded active states of the proteins.

Maturation proteins, which are induced in response to ABA or dehydration, also protect plants under stress by stabilizing cell membranes. Heat shock proteins (Hsps) and molecular chaperones, as well as late-embryogenesis abundant (LEA) protein families, are involved in plant abiotic stress tolerance [20]. High temperature, salinity, and drought stress can cause denaturation and dysfunction of many proteins. Hsps and LEA proteins help to protect against stress by controlling the proper folding and conformation of both structural (i.e., cell membrane) and functional (i.e., enzymes) proteins. Proteins synthesized in response to drought stress are called dehydrins (dehydration induced) and belong to the group II LEA proteins. The dehydrin family of proteins accumulates in a wide range of plant species under dehydration stress, which range in size from 9 to 200 kDa. Dehydrin proteins have been characterized as hydrophilic, heat stable, macromolecular stabilizer, free of cysteine and tryptophan, responsive to ABA, prevent denaturation of cellular proteins, and rich in lysine. They accumulate along with other LEA proteins in response to a particular stress and have been proposed to play an important role in membrane protein stability and osmotic adjustment. A proposed role of dehydrin-like proteins in drought stress has been the protection of cells from dehydration stress. Dehydrin-like proteins may also have a role similar to compatible solutes in osmotic adjustment. Another possible role of stress proteins is to bind with the ions accumulated (ion sequestering) under drought stress and to control solute concentration in the cytoplasm.

33.3.3 Morphological Changes

Plants exposed to sublethal abiotic stress conditions exhibit a broad range of morphogenic responses that include inhibition of cell elongation, localized stimulation of cell division, and alterations in cell differentiation status [21]. As such, abiotic stress stimuli negatively affect plant growth and development through the arrest of the cell cycle machinery. Abiotic stress perception activates signaling cascades that stimulate cell cycle checkpoints, resulting in an impaired G1-to-S transition, slowing down of DNA replication, and delayed entry into mitosis [22]. Water stress induces meristem shortening in leaves of maize and prolongs the cell cycle duration as a result of reduced CDK activity [23]. In plant tissues, water potential and content are maintained close to the unstressed level by increasing uptake or limiting loss, so that loss and uptake rates of water remain balanced. Such a balance is achieved in the short term mainly by developmental and morphological traits, such as stomatal closure that is paralleled by a decreased photosynthetic rate [24]. Indeed, stomatal

closure in response to drought stress restricts CO_2 entry into leaves, thereby decreasing CO_2 assimilation and water loss from the leaves and affecting mesophyll metabolism [25].

In the longer term, the root/shoot ratio, the tissue water storage capacity, the cuticle thickness, and the water permeability are perceived to be the potentially important target traits, of which change in root growth is the most crucial for crop plants to maximize water uptake [26]. Highly water-stressed maize plants respond by rolling the leaf early in the day. The stress-induced morphogenic response is postulated to be part of a general acclimatization strategy, whereby plant growth is redirected to diminish stress exposure [7].

33.3.4 Metabolic Changes

The plant defense response to drought stress is associated with the synthesis of osmoprotectants, osmolytes, or compatible solutes. The accumulation of several organic solutes according to the metabolic responses has drawn much attention.

Solutes that accumulate in the cytosol and do not interfere with enzymatic reactions comprise sugar alcohols (mannitol and sorbitol), the amino acid proline, free amino acids, and sugars in roots and shoots, and glycine-betaine. The synthesis of these compounds by the plant enhances tolerance to drought [27]. Osmoprotectants are small neutral molecules that are nontoxic to the cell at molar concentration and that stabilize proteins and cell membranes against the denaturing effect of stress conditions on cellular functions [28].

The compatible solutes may be classified into two categories: (1) nitrogen-containing compounds such as proline and other amino acids, quaternary ammonium compounds, and polyamines; (2) hydroxy compounds, such as sucrose, polyhydric alcohols, and oligosaccharides [29]. The plant's response to drought is accompanied by the activation of genes involved in the perception of drought stress and in the transmission of the stress signal. One group of genes encodes proteins to protect the cells from the effects of desiccation. These genes include those that govern the accumulation of compatible solutes, passive transport across membranes, energy-requiring water transport systems, and protection and stabilization of cell structures from desiccation and damage by ROS [30]. The second group of genes activated by drought consists of regulatory proteins that further regulate the transduction of the stress signal and modulate gene expression. At least four independent stress-responsive genetic regulatory pathways are known to exist in plants, forming a highly complex and redundant gene network [2, 30]. Two of the pathways are dependent on the hormone ABA, while the other two are ABA independent. These pathways are also implicated in the perception and response to additional stress factors, including cold, high temperature, and salinity.

Mannitol is a major photosynthetic product in many algae and higher plants, enhancing tolerance to water deficit-induced stress primarily through osmotic adjustment [31]. Its mechanisms are likely to involve the scavenging of hydroxyl

radicals (OH^-) and the stabilization of macromolecules [32]. Water deficit alters the synthesis and partitioning of metabolically important carbohydrates in plants. Some of these effects on carbohydrate metabolism might be required for the photosynthetic assimilation of carbon and its conversion to metabolically usable forms. Other stress-induced changes in carbon metabolism might reflect adaptations for stress tolerance [33]. For example, raffinose family oligosaccharides (RFOs), such as raffinose, stachyose, and galactinol, play important roles in the desiccation tolerance of plants. Raffinose accumulates in vegetative tissues under drought stress [34]. RFO biosynthesis requires the presence of galactinol, which is formed by galactinol synthase (GolS) from UDP-Gal and myoinositol. Galactinol is the galactosyl donor for the biosynthesis of raffinose from Suc by raffinose synthase (RafS). Because galactinol has not been assigned any function in plants other than acting as a galactosyl donor for RFOs synthesis, GolS potentially catalyzes a metabolic key step for RFO synthesis. Overexpression of one of them causes an increase in endogenous galactinol and raffinose, as well as an improvement in drought tolerance [35]. Fructans are polyfructose molecules that are soluble carbohydrates and are located in the vacuoles of many plants. Fructan metabolism plays a significant role in drought stress tolerance in plants [36]. As these compounds are soluble, they might play a role in the osmotic adjustment of natural fructan accumulators by varying the degree of polymerization of the fructan pool. Trehalose (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside) is an innocuous, scentless, nonreducing disaccharide and melliferous nonreducing disaccharide containing two glucose residues bound in an α,α-1,1-glycosidic linkage. It is the nonreducing nature of trehalose that determines its high stability to acid, alkali, and heat. Trehalose can become glass state structure by combining two water molecules. Its hydroscopic property is more than three times of sucrose, maltose, glucose, and fructose. In cells, the high tolerance of trehalose to dehydration provides protection to proteins and biomembranes from drying, freezing, and heating. Accumulation of proline is a widespread plant response to environmental stresses, including low water potential. Proline has a clear role as an osmoticum. In particular, because of its zwitterionic, high hydrophilic characteristics, proline acts as a "compatible solute," that is, one that can accumulate to high concentrations in the cell cytoplasm without interfering with cellular structure or metabolism. There is presently no clear agreement on the function of drought-induced accumulation, although a role in osmoregulation seems likely. Other functions of proline accumulation have also been proposed, including statabilization of macromolecules, a sink of carbon and nitrogen for use after relief of water deficit, radical detoxification, and regulation of cellular redox status by proline metabolism [37]. Glycine-betaine, a quaternary ammonium compound, is a very effective compatible solute. In higher plants, glycine-betaine is synthesized from choline (*Cho*) via betaine aldehyde (BA). Glycine-betaine balances the osmotic pressure between outside and inside of cells to cope up with osmotic stress and hence maintains turgor. Moreover, glycine-betaine also protects physiological processes such as photosynthesis and protein synthesis under drought conditions [38].

33.4
Role of ABA in Drought Tolerance

ABA, a plant stress hormone, induces the closure of leaf stomata (microscopic pores involved in gas exchange), thereby reducing water loss through transpiration and decreasing the rate of photosynthesis. These responses improve the water use efficiency of the plant on the short term. ABA plays an important role in seed maturation and dormancy, as well as in the adaptation of vegetative tissues to abiotic environmental stresses such as drought and high salinity [39].

33.4.1
ABA-Dependent Signaling

Drought stress induces *de novo* synthesis of the phytohormone ABA that plays an important role in the adaptation of vegetative tissues to abiotic stresses, such as drought and high salinity, by promoting stomatal closure in guard cells [40]. Many ABA-inducible genes contain a conserved, ABA-responsive, *cis*-acting element, designated ABRE (PyACGTGGC), in their promoter regions. Reversible protein phosphorylation is an early and centrally regulated event in ABA signal transduction, at least in the guard cells. Upon drought stress, the ABA-responsive 42 kDa kinases are activated, thereby phosphorylating the conserved regions of ABA-responsive element binding protein (AREB)/ABFs. Several SNF1-related protein kinases 2 (SnRK2s) such as ABA-activated protein kinase (AAPK) [41] and OST1/SRK2E in *Arabidopsis* [42] were reported as AAPKs. All these kinases phosphorylate *in vitro*, a motif in the so-called "Constant" subdomains found among basic-leucine zipper (b-ZIP) transcription factors (TFs), including AREB1, AREB2, and ABI5 [43]. Some b-ZIP TFs may also be the targets of calcium-dependent protein kinases (CPKs).

Abiotic stress activates the production of intracellular ROS. When the increase in ROS is relatively small, the housekeeping antioxidant capacity is recruited to reset the original balance between ROS production and scavenging, thus reestablishing the redox homeostasis [44]. Otherwise, ROS is sensed by membrane-localized kinases that eventually activate the MAPK (Figure 33.2). MAPK regulates gene expression by altering the TF activity through phosphorylation of serine and threonine residues, whereas ROS is regulated by oxidation of cysteine residues [45]. Changes in gene expression play an important role in plant drought stress response, and many stress-induced genes are known or presumed to play roles in drought resistance. For many of these genes, the hormone ABA is a key signaling intermediate controlling their expression in an ABA-dependent or ABA-independent manner (Figure 33.3), as shown largely by the analysis of ABA-deficient and ABA-insensitive mutants in *Arabidopsis* [46].

33.4.2
ABA-Independent Signaling

The TFs DREB1 and DREB2 (Figure 33.4) are important in the ABA-independent drought-tolerant pathways that induce the expression of stress response genes.

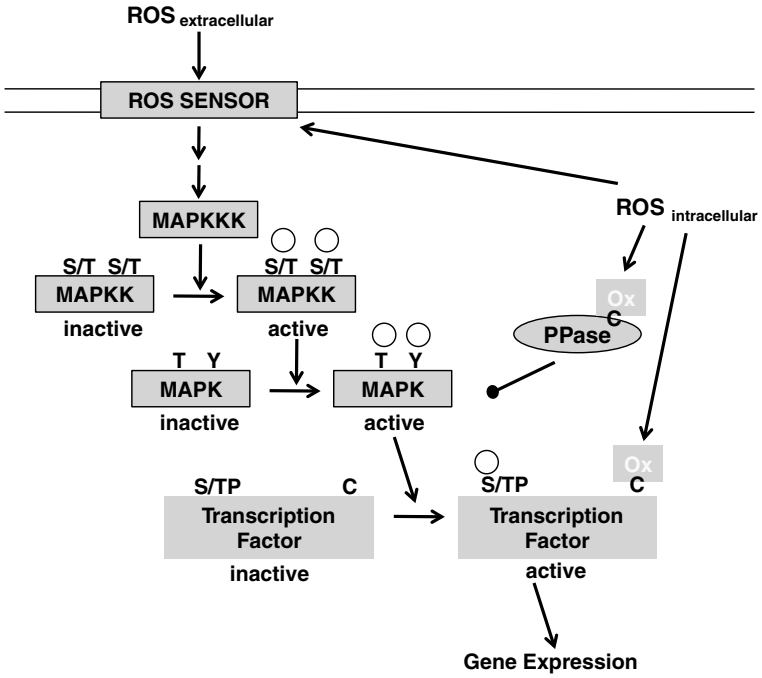

Figure 33.2 Cellular ROS signaling in plants [45].

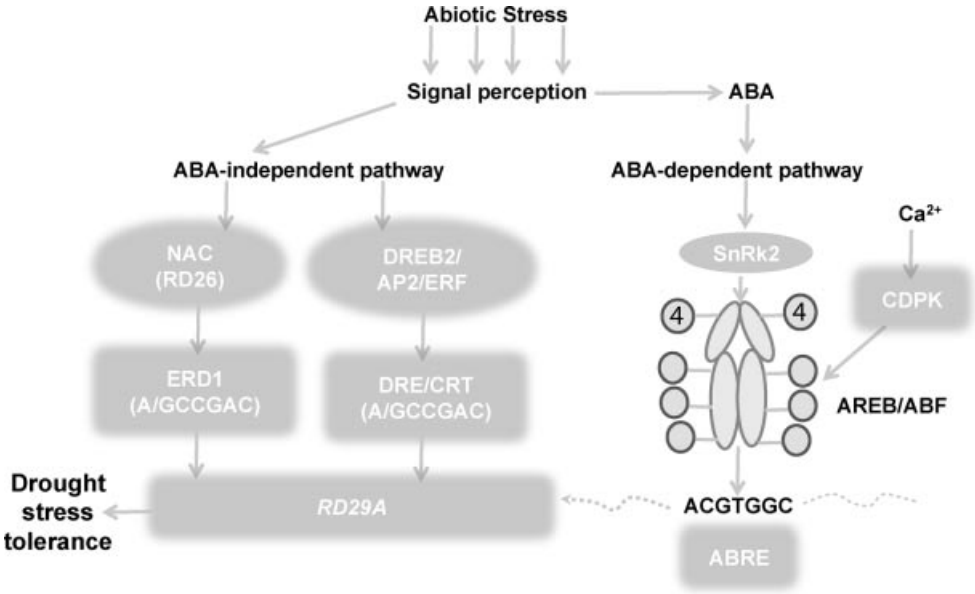

Figure 33.3 ABA-dependent and ABA-independent signaling in plants [46].

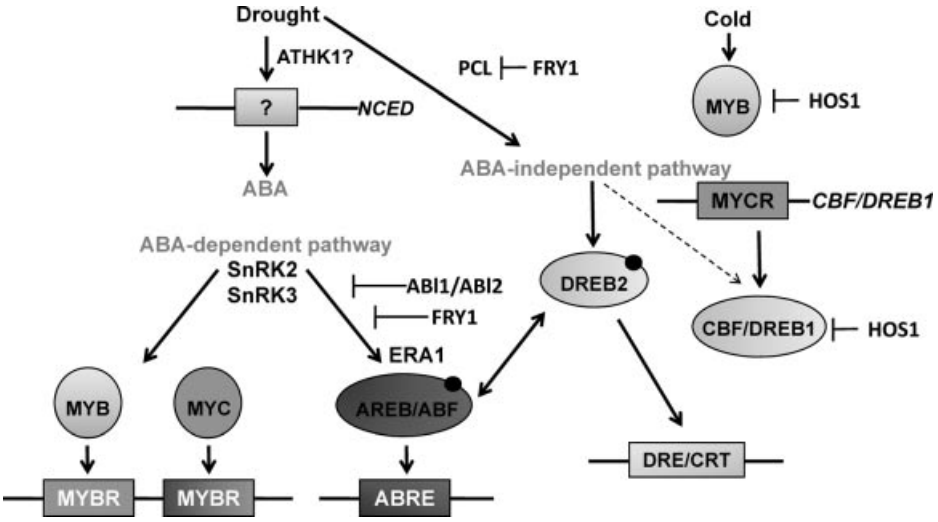

Figure 33.4 Effect of cold, salt loading, and dehydration on expression of stress-related genes leading to striking improvements in plant tolerance [40].

Overexpression of the native form of DREB1, and of a constitutively active form of DREB2, increases the tolerance of transgenic *Arabidopsis* plants to drought, high salinity, and cold. Although these genes were initially identified in *Arabidopsis* plants, their presence and role in stress tolerance have been reported in many other important crops including maize, indicating that this would be a conserved, universal stress defense mechanism in plants.

Abundance of cationic peroxidases induces lignin biosynthesis in the xylem vessels and induces cell wall stiffening that might strengthen the xylem vessels and prevent any further cell expansion either to better withstand the tension occurring during water stress or to restrict water loss from internal tissues [47]. Furthermore, in water-stressed plants, the levels of many amino acids in the sap increase transiently and a number of amino acids also accumulate only under severe water stress. Metabolic changes associated with drought stress include modifications in solute concentration and protein–protein and protein–lipid interactions [48]. Production of phytoalexins, activation of the general phenylpropanoid pathway, and induction of lignin biosynthesis have evolved as adaptation mechanisms to water deficit. Salicylic acid, methyl salicylate, jasmonic acid, methyl jasmonate, and other small molecules produced as a result of stress can also serve as signaling molecules activating systemic defense and acclimatization responses [49], whereas others protect plants from oxidative damage associated with a variety of stresses, such as ascorbic acid, glutathione, tocopherols, anthocyanins, and carotenoids, by scavenging the generated active oxygen intermediates. Knowledge of the physiological and biochemical responses to severe water deficit conditions has been exploited for drought stress tolerance in plants.

33.5
Developing Drought-Tolerant Maize

Unlike relatively static abiotic stresses such as soil salinity or acidity, drought stress in most maize growing areas is strongly dependent upon stochastic weather processes. Transient stresses give rise to complex genotype × year, genotype × season and genotype × season × year, genotype × season × year × management interactions since stress tolerance varies among genotypes and throughout the season of the cropping period. With the advent of combine yield monitors, within-field spatial variation in yield has become much more obvious to the farmer and often relates to variation in soil texture and plant available water.

The application of genetics to improve drought tolerance and provide yield stability is an important part of the solution to stabilizing global maize production. This does not imply that agronomic interventions that aim to maximize water availability at key growth stages are not critically important since genetic solutions are unlikely to close more than 30% of the gap between potential and realized yield under water stress [50]. However, improved genetics can be conveniently packaged in a seed and therefore more easily and completely adopted than improved agronomic practices that depend more heavily on input availability, infrastructure, access to markets, and skills in crop and soil management. Fortunately, under stressful conditions, the performance advantage of modern elite germplasm over its less improved and older counterparts becomes larger, and much of the observed genetic gain in yield during the past 30 years has been attributed to greater stress tolerance rather than to an increase in yield potential *per se* [51–53]. Physiology, coupled with genomics, offers promise of improving the rate of gain for key traits, and especially those such as drought tolerance that are difficult to phenotype, the baseline for comparison must be the rate of improvement obtained through established selection systems. Thus, it is instructive to consider rates of gain in drought tolerance resulting from conventional selection in a large hybrid development program that relies on extensive multi-environment testing to identify superior progenies.

To do this, however, the association between genotype and phenotype must be better understood and quantified so that our ability to predict phenotypic performance from genetic information for many traits observed in an array of environments is greatly improved. Genomics, or the study of the function and structure of specific genetic sequences accompanied by high-throughput laboratory-based analysis of DNA [54], is considered a key to comprehending gene–phenotype associations at the level of candidate genes and sequences. This will be critically important for quantitative traits such as drought tolerance, where performance is regulated by many loci and subject to multiple genotype × environment (G × E), gene × gene (G × G) interactions (epistasis), and gene × gene × environment (G × G × E) interactions.

Identification and measurement of secondary traits associated with grain yield provides a guide to specific mechanisms that contribute to grain yield under drought. Thus, water depletion patterns, leaf rolling, and canopy temperatures are indicative of root exploration and water extraction capacity, and chlorophyll concentration is a

measure of functional stay green [55]. Some secondary traits are associated with specific developmental stages such as flowering, while others, such as photosynthetic rate, are indicative of plant growth throughout the life cycle of the crop. Ideally, secondary traits should be correlated with grain yield under stress, highly heritable, easy to measure, and stable over time. A short anthesis-silking interval (ASI) is indicative of general tolerance to reduced photosynthesis per plant at flowering in many cases [56]. To understand the genotypic differences in water acquisition, a distinction must be made between the fine lateral roots, usually with diameters smaller than 0.8 mm [57], and their larger parental axile roots. In the fibrous root system of maize, axile roots emerge from the stem, guaranteeing a wide vertical and horizontal distribution of the root system, away from the plant basis, while lateral roots are of major importance for the efficient short-distance exploitation of water and nutrients [58]. A series of correlated phenotypes that have associations with grain yield under drought conditions are precocity, plant stature, chlorophyll content, root morphology and conductivity, glucose, sucrose, dehydrin, ABA, and ABA glucose ester measured on leaves, ear tips, and silks harvested at different developmental stages.

Drought tolerance that impacts crop yield can be assessed reliably only in the field. Managed stress environments, where the severity and timing of drought stress are controlled in a manner relevant to target environment conditions, are essential for approaches aimed at achieving genetic progress for drought tolerance. Accurate water management in the absence of rain allows stress intensity to be adjusted so the expression of genetic variability for key secondary traits is maximized and the pattern of stress, targeted at specific growth stages, can be repeated. The detection of genotype $\times$ stress level interactions for drought tolerance provides essential evidence for the presence (and absence) of unique, adaptive mechanisms among genotypes. Generation of such interactions requires the application of relatively severe stress levels that, in some cases, are more severe than those experienced in the target population of environments. A well-watered control is generally needed to monitor for losses in yield potential associated with selection for stress tolerance.

Comparison of performance in these contrasting environments provides the critical data required to predict yield stability of genotypes. Care must be exercised, however, when designing water stress regimes to ensure that the genetic correlation between the managed stress environments and the target population environment remains positive and reasonably large. Under managed stress environments, standard plot management techniques often require adjustment to enhance uniformity within trials. Particularly critical is the establishment of uniform stands to ensure evenness of water availability per plant. As plants remove soil water, differences in root volume per plant and in transpiring leaf area can exaggerate plant-to-plant variability. Blocking by flowering date is important in maize because of its susceptibility to stress imposed at flowering. If entries vary widely in time to silk, the most "tolerant" may simply be those that flower earlier than the mean and thus escape stress that intensifies with time. Finally, time trends in data can occur when variables such as canopy temperature are measured using handheld infrared thermometers [59]. As soil water is depleted, spatial variability generated by differences in

soil texture becomes increasingly obvious and can obscure genotypic differences. The use of uniform land is obviously the best solution, and knowledge of these patterns generated over time can be used to select the most uniform plot sites. The use of incomplete block designs such as row/column or alpha (0, 1) designs [60] or augmented designs provides a method to adjust data for the effects of within-replicate spatial variation when dealing with large entry numbers [55]. Spatial trends in data arise from soil heterogeneity as well as unintentional management factors that can be identified within a linear mixed model analysis framework. In addition, data can be adjusted for these effects by incorporating appropriate model terms and identifying appropriate variance structures for spatial trends [61]. Linear mixed models can be used for estimating variance components and determining best linear unbiased predictors (BLUPs) for genotypes from unbalanced data sets.

33.6
Modern Tools to Improve Drought Tolerance in Maize

Integrating molecular approaches of the latest advances in biotechnology, genomic research, and molecular marker applications with conventional plant breeding, plant physiology, and biochemistry could increase significantly the potential for genetic gain under water-limited conditions (Figure 33.5).

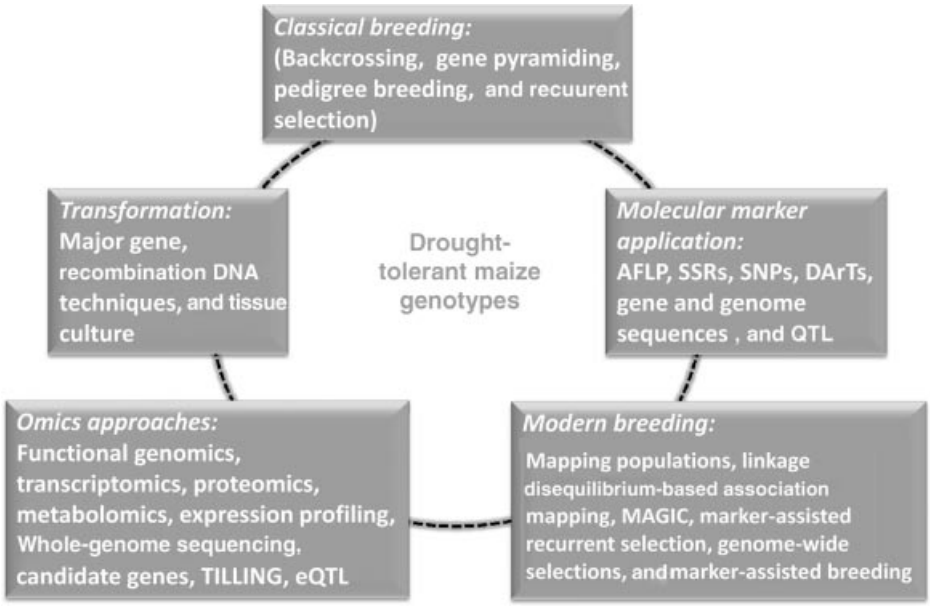

Figure 33.5 Development of drought-tolerant maize genotypes by integration of classical and modern tools.

Changing maize genetic improvement from an empirical to a knowledge-based process involves investing heavily in the use of high-throughput recombinant DNA technology, genomics, and bioinformatics tools. The utility of these tools has, in turn, been increased by advances in DNA sequencing capacity and in database development and management. The availability of organized dense genetic maps based on molecular markers and the awareness of the particulate nature of the inheritance of quantitative traits have fostered an interest in the genetic dissection of drought tolerance. Ideally, this involves associating genetic variation at the sequence level with observed phenotypic variation and ensuring that those specific sequences imparting tolerance are present in subsequent generations.

Genetic mapping with dense marker maps can be used to identify the number and genetic positions of quantitative trait loci (QTL) associated with a specific trait under drought stress. In addition, this process can be used to estimate effects of the segregating QTL and their contributions to trait variation (individually and in combined QTL models) and to obtain estimates of their stability across environments (QTL × environment interactions) and across genetic backgrounds (QTL × genetic background interactions).

The responses of the genotypes to drought stress are governed by the activity of several genes involved in diverse pathways, including "constitutive" QTL and "adaptive" QTL. DNA-based markers located in such genomic (bin) locations could potentially serve as informative "anchor" markers for molecular marker-assisted selection (MAS) as well as functional genomics. In addition to the consensus QTL, analysis of individual genes, transcriptome profiling, and *in silico* mapping leads to identification of specific candidate genes with significant influence over drought stress tolerance in maize, many of which colocalize with the consensus QTL for drought tolerance. Lebreton *et al.* [62] were the first to attempt to apply QTL analysis to obtain genetic insights into the drought tolerance response in maize. Since then, a number of reports of QTL associated with specific traits under drought stress have been indicated using diverse mapping populations [63–66]. The reports have targeted grain yield and its components, ASI, root traits, and measures of plant water use and status, such as stomatal conductance, and leaf and xylem ABA content [67].

Progress of trait is attributed to the number of major QTL identified per trait, the magnitude of observed phenotypic variance that they generally express individually, their interaction with the environment, and difficulty of epistasis evaluation [68]. For complex traits such as drought tolerance, many QTL identified in elite lines developed by breeding programs are likely to be context dependent due to the effects of several gene and environmental interactions. Therefore, although we can mendelize quantitative traits, the value of the QTL alleles will need to be determined for the specific situation to which they are to be applied. Capacity for precision phenotyping under repeatable but representative levels of stress in the field is lagging far behind the capacity to generate genomic information and will limit progress in generating gene–phenotype associations for traits.

MAS experiments based only on the QTL involved in the expression of yield components would be inefficient because only a few of the QTL are stable across environments. A MAS experiment should consider the QTL involved in the expres-

sion of secondary traits of interest correlated with the yield under drought. If it is possible, the selected QTL should be stable across environments and account for a large percentage of the phenotypic variance. Therefore, an efficient MAS strategy should take into account the most suitable QTL from different traits as an index [69]. Many studies clearly indicate the colocation of the QTL influenced including key traits such as grain yield, ASI, root traits, and ABA accumulation across different genetic backgrounds [5]. In maize, MAS has been used to introgress QTL alleles for reducing ASI.

Compared to modern cultivars, which are usually selected for high-input environments where water supply is often not a major limiting factor, wild species show morphophysiological features for survival and adaptation to drought conditions. When considering exploiting alleles from wild species for drought adaptive features, a careful evaluation in terms of yield, once they are backcrossed in elite accessions, is important [68].

Information about QTL in tropical maize can be applied to increase heritability and favorable gene action to design optimum transgenic strategies for crop improvement. Marker-assisted selection also accelerates the use of transgenes in commercial cultivars, typically achieved through marker-assisted backcrossing [70]. An updated compilation of mapped QTL and major genes associated with abiotic stress tolerance including drought in maize and other plants is available at www.plantstress.com [71]. Other useful Web resources are www.generationcp.org, http://rarge.gsc.riken.jp/, and http://rootgenomics.missouri.edu/ [5]. Identification of universal drought QTL and putative candidate genes could be valuable for further analysis and utilization [72].

33.7
Functional Genomics of Drought Tolerance

Functional genomics uses mostly multiplex techniques to measure the abundance of many or all gene products such as mRNAs or proteins within a biological sample. Functional genomics includes function-related aspects of the genome itself such as mutation and polymorphism (e.g., single-nucleotide polymorphic (SNP)) analysis, as well as measurement of molecular activities. The latter comprise a number of "omics" such as transcriptomics (gene expression), proteomics (protein expression), phosphoproteomics (a subset of proteomics), and metabolomics (analysis of metabolites).

Functional genomics provides important information for evaluating stress perception, signal transduction, and defensive responses, the role of potential candidate genes, and the pathways in which they are involved. Intensive studies have already led to the discovery of promoter regulatory elements, such as DRE (dehydration-responsive elements) or ABRE (ABA-responsive elements), involved in both dehydration and low-temperature-induced gene expression [73], as well as identification of several key transcriptional factors interacting with such promoters [74]. With the advent of genomics-related technologies, necessary tools to identify the key gene

networks that respond to drought stress and those relating to the regulation of adoptive events occurring during stress are now becoming available [75].

Cloning a drought-related QTL substantially contributes toward a better understanding of the genetic and functional basis of the response of a plant to drought. Furthermore, the sequence responsible for the QTL becomes available for genetic engineering and mining for the most desirable alleles within germplasm collections. Until now, two approaches have been mainly used for the molecular dissection of a QTL: positional cloning and association mapping [76].

Positional cloning begins with the production of a large population in a near-isogenic lines (NILs) where only the target QTL segregates. A large number of progenies (>1000) capturing all possible recombination events and molecular markers in the target region enable us to identify the genetic and physical interval cosegregating with the QTL. The availability of the genome sequence facilitates the connection between genetic and physical information. When the genome sequence is not available, genomic libraries (e.g., BAC clones) are used. A maize domestication locus, teosinte glume architecture (*tga1*), which encodes a transcriptional regulator, was the first maize gene positionally cloned, using a population of over 3000 individuals [77]. Among the quantitative traits affecting drought tolerance, particular attention has been devoted to the concentration of ABA, in view of its pivotal role in regulating other molecular and morphophysiological processes involved in the adaptive responses. Differences among NIHs (near-isogenic hybrids) for leaf ABA and other morphophysiological traits were not affected by water regimes. On average, the QTL allele for high leaf ABA markedly reduced stomatal conductance and root lodging [78]. Candidate genes or sequences that cosegregate with the QTL are then functionally tested with reverse genetics tools (e.g., knockout mutants, RNAi, and targeting induced local lesions in genomes (TILLING)) and ectopic expression.

Association mapping based on linkage disequilibrium (LD) seeks to establish a statistical association between allelic (or haplotype) variation at a locus and the phenotypic value of a trait across a large enough sample of unrelated accessions [79]. The LD approach offers two distinct advantages: the survey of multiple alleles in a single analysis and avoidance of the time-consuming preparation of mapping populations. In the presence of high LD (~100 kb or more), association mapping can provide only coarse mapping information; however, when LD is low (~10 kb or less), the resolution power is sufficiently high to assign a QTL to an interval containing one or a few genes. Analysis of candidate genes has already provided interesting results [79, 80]. Association mapping should greatly benefit from the introduction of high-throughput platforms that are able to profile a multiplex of SNP markers. Techniques such as EcoTILLING [81] are also available to streamline the identification and scoring of new alleles at target genes or sequences.

Major challenges for gene discovery include the following:

- The large size of the maize genome
- Variation in genome size and gene order
- The high incidence of multicopy genes
- Transposons and other repetitive sequences making up a large portion of the genome

The wealth of active transposable elements residing in the maize genome plays an important role in functional genomics. In addition to serving as molecular tags for mutated genes, these transposons tend to knock out genes into which they are inserted. Mutant libraries constructed using transposon tagging, T-DNA insertion, and chemical and physical mutagenesis provide materials that can be screened for base changes in any genes by reverse genetics methods such as TILLING [82].

Strategies have been developed so that subtle changes such as point mutation generated by EMS can be detected easily. In the basic TILLING method, pollen is mutagenized by treatment with EMS. These mutagenized lines serve as a general reverse genetic resource. Weil and Monde [83] provided a detailed protocol for maize TILLING including TILLING mutagenesis, tissue collection, DNA preparation, 2D pooling, and detailed TILLING workflows. As the maize genome is completely sequenced, advances in reverse genetics technologies including TILLING, EcoTILLING, and massively parallel DNA resequencing provide excellent methods for identifying mutations in a wide variety of traits and biological processes [84]. EcoTILLING is a way to survey how much allelic diversity there is for a given gene target and where that diversity is located (intron versus exon).

Gene expression experiments have identified several hundred genes that are induced or repressed during drought. Both cDNA- and oligonucleotide-based gene expression profiling platforms are used to examine the effects of drought stress on yield potential of maize [85]. The expression QTL (eQTL) mapping involves expression profiling as measured by mRNA transcript abundance for a large number of genes that are each treated as a quantitative phenotype likely to be conveyed by multiple genes and influenced by environmental factors. These expressional profiles then constitute a marker-based fingerprint of each individual in a segregating population and can be subjected to conventional QTL analysis [86], albeit interpreted in the spatially and temporally specific context in which the data are collected. The procedure is called mapping of eQTL, that is, genetic locus where allelic variation affects the level of gene expression. Overlapping expression profiles and coordinate expression indicated that genes relevant to stress resistance and such data sets form an excellent resource for identifying candidate genes [87] through positional cloning or association mapping. Transcription profiling has increasingly become an important genomics tool for gene functional analysis. Expression patterns of some genes in several stress response-associated pathways, including abscisic acid, jasmonic acid, and phenylalanine ammonia lyase, are positively responsive to drought stress. However, the cost of profiling the large number of samples required to identify eQTL is still too high for routine application of this approach. The expression is most often quantified in terms of the amount of mRNA in a microarray-based analysis, but the same principle has been applied to genetic control of the protein level. Microarrays have become an important technology for the global analysis of gene expression. Implemented in the context of a well-designed experiment, cDNA and oligonucleotide arrays can provide high-throughput simultaneous analysis of transcript abundance for hundreds, if not thousands, of genes. Microarrays are being used to assess gene expression in plants exposed to the experimental manipulation of air temperature and soil water content in the root zone. Analysis often includes

characterizing transcript profiles for multiple posttreatment sampling periods and categorizing genes with common patterns of response using hierarchical clustering techniques. In addition, microarrays are also providing insights into developmental changes in gene expression associated with root elongation in maize [88].

The identification of candidate genes for the QTL and the elucidation of their functional role can be facilitated by combining QTL maps with the so-called functional maps (i.e., maps enriched with genes potentially involved in controlling the target trait or with fully annotated genomic sequences). An important part of new gene discovery through EST and genome sequencing is the annotation of those genes to assign putative functions [89]. In the absence of empirical data for a particular gene in the appropriate target organism, gene annotation software can predict a function using data from rice, *Arabidopsis*, and other organisms based on similarities for intron/exon sequence and structure plus likely protein domains (www.maizesequence.org). One can BLAST maize sequences against the cereal and other species databases to generate predicted functional information. In forward and reverse genetics approaches, maize is characterized by excellent mutagenesis resources in the form of well-studied transposon systems, and new techniques for induced mutations are also being applied [90].

Maize is one of the model systems for genetic research. The genome size of the maize is 2300 Mb, which is fivefold larger than rice but eightfold smaller than wheat [91]. The sequencing of maize was recently completed by a U.S.-based consortium of researchers [92]. The complete genome of B73, an important commercial crop variety, was decoded. The 2.3 billion base sequence – the largest genetic blueprint worked out for any plant species – includes more than 32 000 protein-coding genes spread across 10 chromosomes of maize. The transposable elements are the most abundant parts of the sequence, spanning almost 85% of the genome and dispersed nonuniformly across the genome. The Maize Genome Sequencing Consortium has generated a reference genome sequence that was integrated with both physical and genetic maps. Using a previously published integrated genetic and physical map, combined with genomic sequence, new sequence-based genetic markers, and an optical map, the researchers have picked a minimum tiling path (MTP) of 16 910 bacterial artificial chromosome (BAC) and fosmid clones that were used to sequence the maize genome. The new integrated physical and genetic map covered 2120 Mb (93%) of the 2300 Mb genome, of which 405 contigs were anchored to the genetic map, totaling 2103.4 Mb (99.2% of the 2120 Mb physical map). Using all available physical, sequence, genetic, and optical data, a golden path (AGP) of chromosome-based pseudomolecules, referred to as the B73 reference genome sequence version, was generated [93].

The completion of the maize genome sequence provides the most essential resource to move easily from gene to mutant phenotype and back. There are several methods such as gene cloning, gene expression profiling, TILLING/EcoTILLING and transposon tagging, and SNP haplotypes to target loci for experimentally determining gene function [94]. Traditionally, gene discovery in maize has employed transposon tagging, EST searches, and comparative genomics, and due to increase in genomic resources, positional cloning is increasingly being used for both qualitative

and quantitative traits. Now with the physical maps for maize, the large numbers of available markers, and conservation of synteny across the cereal genomes, it is feasible to consider a less time-consuming chromosome walk rather than cloning by transposon tagging [95].

The length and breadth of the utility of genome sequencing in crop research has increased with the availability of new-generation sequencing (NGS) technologies. This new-generation techniques will increase the throughput tremendously while reducing the cost multiple times [96, 97]. The data generated through NGS techniques have opened new era of maize genome analysis and provide sequence information of the germplasm that are genetically diverse so as to uncover the genetic potential of the unexplored genotypes. Getting enormous amount of data cheaply in no time has extended its applications beyond just reading the order of bases. Microarrays are being replaced by sequence-based method in gene expression studies. The ability to sequence the whole genome of many organisms will allow large-scale comparative and evolutionary studies [98]. Sequencing technology is developing very rapidly and already the third-generation sequencing platforms are being made available such as "real-time sequencing" (www.pacificbiosciences.com).

Genomics and bioinformatics allow us to investigate sequence colinearity in the main crops [99] and compare their gene order and content with those of model species whose genomes have been sequenced, such as rice and *Arabidopsis*. The colinearity between *Arabidopsis* and maize [100] has been eroded to such an extent that the *Arabidopsis* sequence does not appear to be of much help for the identification of related genes in maize. Conversely, comparative mapping between rice and maize [101] as well as other cereals [102] provides valuable opportunities to exploit high-resolution collinear maps to facilitate the positional cloning of maize QTL and identify candidate genes and to establish whether sequences with high homology are so because they represent orthologous loci.

Transcriptomics, proteomics, and gene expression studies have identified the activation and regulation of several stress-related transcripts and proteins that are generally classified into two major groups. One group is involved in signaling cascades and in transcriptional control, whereas members of the other group function in membrane protection, as osmoprotectants, as antioxidants, and as ROS scavengers [103]. Manipulation of genes that protect and maintain cellular functions or that maintain the structure of cellular components has been the major target of attempts to produce plants that have enhanced stress tolerance [104].

Progress in the mass-scale profiling of the transcriptome, proteome, and metabolome has allowed a more holistic approach in investigations of drought tolerance based on the measurement of the concerted expression of thousands of genes and their products. High-throughput mRNA profiling has been applied to investigate the changes in gene expression in response to dehydration [105]. An example of how transcriptome analysis can advance our understanding of the physiology underpinning drought-related traits has been recently provided by the expression profiling of primary root apices in maize [106]. Collectively, the transcriptome profiling experiments conducted on drought-stressed plants have highlighted the central role of TFs while unveiling the complex hierarchy of the regulatory network that differentially

modulates the expression of dehydration signature genes in a tissue-specific manner. In this respect, laser captured microdissection is a major technical breakthrough: the technique allows the profiling of specific cell types [107] – a feature particularly important when investigating genes encoding for TFs expressed weakly and in a cell type-specific fashion.

The importance of metabolic changes during plant responses to abiotic stress suggests that detailed metabolite profiling may provide valuable insights into stress response mechanisms. Deciphering gene function can also be facilitated by information gathered through profiling the proteome and metabolome. Profiling the proteome of a mapping population offers the opportunity to identify PQL (protein quantity loci) influencing protein quantity. In water-stressed maize, the *Asr1* gene, a putative TF, has been shown to colocalize with a PQL for the ASR1 protein and a QTL for ASI and leaf senescence [108]. With regard to metabolomics, the present technology enables the profiling of ~2000 metabolites in a single sample [109]. The susceptibility of early developing grain to water stress is a major problem in maize, where a shortage of assimilate supply has been indicated as the likely cause for insufficient grain filling and sterility [110]. In this respect, invertase activity in the developing kernel has been shown to be an important limiting factor for grain yield in maize exposed to drought [111]. Among the QTL for invertase activity described in maize, one is mapped near *Ivr2*, an invertase-encoding gene [112]. Furthermore, collocation between the activities of two enzymes (sucrose-P synthase and ADP-glucose pyrophosphorylase) involved in carbohydrate metabolism and corresponding structural genes has been reported in young maize plants subjected to water deficit [113]. The increase in *Hsp* expression under conditions of abiotic stress was studied extensively using functional genomics and proteomics in different plant species [114]. Genomics-based approaches can contribute novel information to identify candidate genes and elucidate their functions and regulation under water-limited conditions.

33.8
Genetic Engineering Approaches for Improving Drought Tolerance

Although not a crop plant, *Arabidopsis* has played a vital role in the elucidation of the basic processes underlying stress tolerance, and the knowledge obtained has been transferred to a certain degree to important food plants. Many of the genes known to be involved in stress tolerance have been isolated initially from *Arabidopsis* [115]. Two general strategies for the metabolic engineering of abiotic stress tolerance have been proposed: increased production of specific desired compounds or reduction in the levels of unwanted (toxic) compounds [116]. However, modulation of a single enzymatic step is usually regulated by the tendency of cell systems to restore homeostasis, thus limiting the potential of this approach. Targeting multiple steps in the same pathway could help to control metabolic fluxes in a more predictable manner [117]. Effort heavily depends on the development and utilization of drought-tolerant germplasm resources, which is far from plentiful and bottlenecks maize

improvement. Therefore, germplasm enhancement and development is foundationally important for maize improvement [118, 119]. Transgenic operation is a useful technology to overcome reproductive isolation among species and utilize beneficial exotic genes.

Acquired plant tolerance to abiotic stress can be achieved both by genetic engineering and by conventional plant breeding combined with the use of molecular markers utilizing QTL, Hsp, LEA, and ROS (Figure 33.6). The tolerance of the transgenic offspring is not strong enough to meet the requirement of maize production, as the mechanism of these exotic genes is not adaptive to physiological metabolism of maize. It is the key step to explore new exotic gene with strong tolerance and adaptive mechanism to maize [121–123]. Seed of improved cultivars has shown itself to be an effective means of delivering conventional and transgenic traits that contribute to improved yield and its stability.

Many loci for genes that control tolerance to abiotic stress in plants have been identified by genetic analysis [124, 125]. However, many genes that control agronomically important traits remain to be identified and modified to generate new varieties with desirable traits. There is evidence that transgenic plants in which the expression of a single gene has been modified have enhanced tolerance to abiotic stress [126]. Ideally, modification of a single gene should confer tolerance to more than one form of abiotic stress [127].

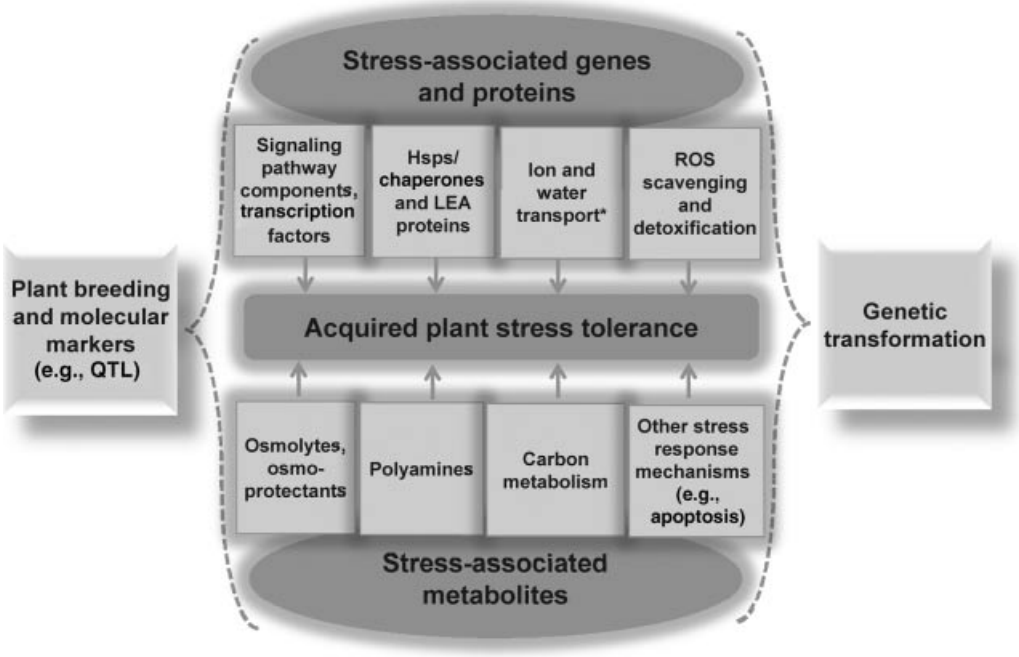

Figure 33.6 Approaches to improve stress tolerance [120].

SOD, dehydration-responsive element binding (DREB), and some other stress-tolerant genes have been used to transform maize for drought tolerance improvement. A new sequence of trehalose synthase gene *TPS1* was cloned from *Saccharomyces cerevisiae* using the method of homologous amplification. Sequence analysis showed that its similarity to formerly reported sequence of gene *TPS1* was as high as 99.3%. The putative protein of this sequence had the same conserved contigs with the protein sequences of trehalose synthases in many eukaryotic and prokaryotic organisms. This sequence was used as exotic gene to construct a stress-inducible expression vector and transform embryonic calli of maize mediated by *Agrobacterium*. After screening and regeneration, one fertile plant was detected to be positive using specific PCR amplification and sequencing of the amplified product [128]. Quan *et al*. [129] transformed maize with *betA* gene from *Escherichia coli* encoding choline dehydrogenase, a key enzyme in the biosynthesis of glycine-betaine. The transgenic maize plants accumulated higher levels of glycine betaine and were more tolerant to drought stress than wild-type plants (nontransgenic) at germination and the young seedling stage [130].

Multiple transgenic approaches include the use of polyol compounds such as mannitol [131] and sorbitol [132]; dimethylsulfonium compounds such as dimethylsulfoniopropionate and glycine-betaine [127]; sugars such as sucrose, trehalose [133], galactinol [34], ononitol [134], and fructan [135]; or amino acids such as proline [136] and ectoine that serve as osmolytes and osmoprotectants [7]. Therefore, it has been hypothesized that engineering the introduction of osmoprotectant synthesis pathways is a potential strategy for improving the stress tolerance of crop plants [48]. The genetic engineering of metabolic pathways for the production of osmolytes, such as mannitol, fructans, trehalose, proline, and glycine betaine, among others, might increase resistance to drought, but the mechanism by which these osmolytes provide protection is not completely understood [137]. Usually, the osmolytes are localized in the cytoplasm of plants. The active accumulation of osmolytes decreases the osmotic potential of cell and maintains cell turgor. Other responses, such as the production of scavenging ROS and the induction of chaperone-like activities that protect protein structure and metabolic detoxification, are also being reported during drought stress [138].

Metabolic engineering has allowed the introduction of biosynthetic pathways of glycine-betaine from microorganisms into maize. Indeed, maize accumulated higher levels of glycine-betaine when transformed with the beta gene from *E. coli* that encodes choline dehydrogenase, a key enzyme in the biosynthesis of glycine-betaine [129]. An assortment of genes with diverse functions are induced or repressed by these stresses [139]. Most of their gene products may function in stress response and tolerance at cellular level. Significantly, the introduction of many stress-inducible genes via gene transfer resulted in improved plant stress tolerance [2, 140].

Genetic engineering of plants for tolerance to extreme abiotic stresses could be achieved by the regulated expression of stress-induced TFs, which, in turn, would regulate the expression of a large number of relevant downstream genes [141]. TFs have been used to elicit multiple biochemical and developmental pathways that regulate drought tolerance, thereby improving performance during drought under

laboratory and greenhouse conditions [2]. The best stress-responsive TFs are the C-repeat binding factor (CBF)/dehydration-responsive element binding proteins that belong to the AP2/ethylene-responsive element binding protein family [142]. These factors enhance or modulate the expression of genes with a CBF/DRE box in their promoters and define a major stress tolerance pathway, in addition to the ABA biosynthesis/response pathway. On the other hand, the promoter of the early response to dehydration 1 (*ERD1*) gene contains *cis*-acting element(s) involved in ABA-independent stress-responsive gene expression [143]. Positive reports on the use of TFs to improve drought resistance in model and crop plants are based on laboratory and greenhouse conditions rather than field conditions; hence, the use of TFs in enhancing drought tolerance in crop plants should be considered with caution. Therefore, there is a need for understanding the basic molecular mechanisms influencing drought tolerance and grain yield under field stress conditions [7]. Till date, various genes and TFs crucial for stress tolerance have been studied [144].

Recently, drought tolerance in transgenic maize plants under field conditions has been enhanced through overexpression of NF-YB [144], which is part of a ubiquitous TF composed of three distinct subunits, NF-YA (HAP2), NFYB (HAP3), and NF-YC (HAP5) [145]. The NF-Y complex is also known as the HAP or the CAAT complex that acts in concert with other regulatory factors to modulate gene expression in a highly controlled manner. The overexpression of a maize CAAT box TF (ZmNFYB2) imparted significant tolerance to drought, resulting in increased yield. Most important, in field trials, the transgenic lines gave higher grain yields than control lines under drought conditions [7]. Engineering upstream signaling components of drought stress pathways might be another promising way to obtain drought stress tolerance. Indeed, constitutive expression of NPK1, a tobacco MAPKKK, in maize enhanced drought tolerance, as demonstrated by higher photosynthesis rates and higher kernel weight in the transgenic plants than those of the nontransgenic controls under greenhouse dehydration conditions [146]. The stress adaptation responses contribute to a yield advantage in maize that is grown within drought environments. The application of this technology is therefore expected to have the most significant impact on severely water-limited maize production systems [144].

33.9 Conclusions

The occurrence of drought varies unpredictably according to years, seasons, places, and within fields, so maize genotypes able to withstand stress throughout their life cycles at no cost to yield potential are the need of the hour. The use of genetics and genomics within an integrated framework that relies heavily upon critical input from disciplines such as plant breeding, crop physiology, crop modeling, and precise field phenotyping is sought. This integration of quantitative knowledge arising from diverse, but complementary, disciplines will allow researchers to more fully understand genes associated with drought tolerance in maize and more accurately predict the consequences of modulating expression levels of those genes.

Knowledge-based approach can improve maize production for the drought-prone weather and to develop more focused field screening techniques that increase rates of gain for yield and its stability under conditions of variable and unpredictable water stress. It is thus essential to test newly developed genotypes to multiple stresses and to carry out extensive field studies under a large range of conditions that assess tolerance as absolute yield increases. As a number of measures are in place to ensure the safe and responsible design of field tests, especially the transgenic approach, excessive precaution should not become a barrier to using all the tools available to us for a more sustainable agriculture.

References

1 Azeez, J.O., Chikoye, D., Kamara, A.Y., *et al.* (2005) *Plant Soil*, **276**, 61–68.
2 Umezawa, T., Fujita, M., Fujita, Y., *et al.* (2006) *Curr. Opin. Biotechnol.*, **17**, 113–122.
3 Salekdeh, G.H., Reynolds, M., Bennett, J., *et al.* (2009) *Trends in Plant Sci.*, **14**, 488–496.
4 Edmeades, G.O., Baezinger, M., Mickelson, H.R., *et al.* (1997) Recent advances in breeding for drought tolerance in maize, in *Contributing to Food Self-Sufficiency: Maize Research and Development in West and Central Africa* (eds B. Badu-Apraku, M.O. Akoroda, M. Duedraogo, and F.M. Quin), IITA, Cotonou, Benin Republic, p. 404 (Proceedings of a Regional Maize Workshop, May 29–June 2, 1995, IITA, Cotonou, Benin Republic).
5 Tuberosa, R. and Salvi, S. (2006) *Trends Plant Sci.*, **11**, 405–412.
6 Kramer, P.J. and Boyer, J.S. (1995) *Water Relations of Plants and Soils*, Academic Press, San Diego.
7 Anami, S., De Block, M., Machuka, J., *et al.* (2009) *Crit. Rev. Plant Sci.*, **28**, 16–35.
8 Rhoads, F.M. and Bennet, J.M. (1990) Corn, in *Irrigation of Agricultural Crops* (eds B.A. Stewart and D.R. Nielsen), American Society of Agronomy, Madison, pp. 569–597.
9 Denmead, O.T. and Shaw, R.H. (1960) *Agron. J.*, **52**, 272–275.
10 Schussler, J.R. and Westgate, M.E. (1995) *Crop. Sci.*, **35**, 1074–1080.
11 Zinselmeier, C., Westgate, M.E., Schussler, J.R., *et al.* (1995) *Plant Physiol.*, **107**, 385–391.
12 Westgate, M.E. (1997) Physiology of flowering in maize: Identifying avenues to improve kernel set during drought, in *Developing Drought and Low-N Tolerant Maize* (eds G.O. Edmeades, *et al.*), CIMMYT, El Batan, Mexico, pp. 136–141.
13 Bolanos, J. and Edmeades, G.O. (1996) *Field Crop. Res.*, **48**, 65–80.
14 Araus, J.L., Slafer, G.A., Reynolds, M.P., *et al.* (2002) *Ann. Bot.*, **89**, 925–940.
15 Langridge, P., Paltridge, N., and Fincher, G. (2006) *Brief. Funct. Genomic. Proteomic.*, **4**, 343–354.
16 Wasilewska, A., Vlad, F., Sirichandra, C., *et al.* (2008) *Mol. Plant.*, **1**, 198–217.
17 Nanjo, T., Kobayashi, M., Yoshiba, Y., *et al.* (1999) *FEBS Lett.*, **461**, 205–210.
18 Holmberg, N. and Bűlow, L. (1998) *Trends Plant Sci.*, **3**, 61–66.
19 Shen, B., Jensen, R.G., and Bohnert, H.J. (1997) *Plant Physiol.*, **113**, 1177–1183.
20 Wang, W., Vinocur, B., Shoseyov, O., *et al.* (2004) *Trends Plant Sci.*, **9**, 244–252.
21 Potters, G., Pasternak, T.P., Guisez, Y., *et al.* (2007) *Trends Plant Sci.*, **12**, 98–105.
22 Kadota, Y., Goh, T., Tomatsu, H., *et al.* (2004) *Plant Cell Physiol.*, **45**, 160–170.
23 Granier, C., Inze, D., and Tardieu, F. (2000) *Plant Physiol.*, **124**, 1393–1402.
24 Lawlor, D.W. and Cornic, G. (2002) *Plant Cell Environ.*, **25**, 275–294.
25 Parry, M.A.J., Androlojc, P.J., Khan, S., *et al.* (2002) *Ann. Bot.*, **89**, 833–839.

26 Verslues, P.E., Agarwal, M., Katiyar-Agarwal, S., *et al.* (2006) *Plant J.*, **45**, 523–539.

27 Riccardi, F., Gazeau, P., Jacquemot, M.P., *et al.* (2004) *Plant Physiol. Biochem.*, **42**, 1003–1011.

28 Yancey, P.H. (1994) Compatible and counteracting solutes, in *Cellular and Molecular Physiology of Cell Volume Regulation* (ed. K. Strange), CRC Press, Boca Raton, pp. 81–109.

29 McCue, K.F. and Hanson, A.D. (1990) *Trends Biotechnol.*, **8**, 358–362.

30 Shinozaki, K. and Yamaguchi-Shinozaki, K. (2007) *J. Exp. Bot.*, **58**, 221–227.

31 Loescher, W.H., Tyson, R.H., Jacquemot, M.P., *et al.* (1992) *Plant Physiol.*, **98**, 1396–1402.

32 Shen, B., Jenson, R.G., and Bohnert, H.J. (1997) *Plant. Physiol.*, **115**, 527–532.

33 Pattanagul, W. and Madore, M.A. (1999) *Plant Physiol.*, **121**, 987–993.

34 Taji, T., Ohsumi, C., Iuchi, S., *et al.* (2002) *Plant J.*, **29**, 417–426.

35 Amiard, V., Morvan-Bertrand, A., Jean-Pierre, B., *et al.* (2003) *Plant Physiol.*, **132**, 2218–2229.

36 Vereyken, I.J., Chupin, V., Islamov, A., *et al.* (2003) *Biophys. J.*, **85**, 3058–3065.

37 Mohammadkhani, N. and Heidari, R. (2008) *World Appl. Sci. J.*, **3**, 448–453.

38 Sarwar, M.K.S., Ullah, I., Urrahman, M., *et al.* (2006) *Pak. J. Bot.*, **38**, 1449–1456.

39 Ortiz, R., Iwanaga, M., Reynolds, M.P., *et al.* (2007) *J. SAT. Agr. Res.*, **40**, 1–30.

40 Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M. (2003) *Curr. Opin. Plant Biol.*, **6**, 410–417.

41 Li, W.X., Oono, Y., Zhu, J., *et al.* (2008) *Plant Cell*, **20**, 2238–2251.

42 Mustilli, A.C., Merlot, S., Vavasseur, A., *et al.* (2002) *Plant Cell*, **14**, 3089–3099.

43 Furihata, T., Maruyama, K., Fujita, Y., *et al.* (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 1988–1993.

44 Foyer, C.H. and Noctor, G. (2005) *Plant Cell*, **17**, 1866–1875.

45 Apel, K. and Hirt, H. (2004) *Ann. Rev. Plant Biol.*, **55**, 373–399.

46 Koornneef, M., Léon-Kloosterziel, K.M., Schwartz, S.H., *et al.* (1998) *Plant Physiol. Biochem.*, **36**, 83–89.

47 Alvarez, S., Marsh, L.E., Schoeder, S.G., *et al.* (2008) *Plant Cell Environ.*, **31**, 325–340.

48 Valliyodan, B. and Nguyen, H.T. (2006) *Plant Biol.*, **9**, 1–7.

49 Shulaev, V., Cortes, D., Miller, G., *et al.* (2008) *Physiol. Plant*, **132**, 199–208.

50 Edmeades, G.O., Banziger, M., Schussler, J.R., *et al.* (2004) Improving abiotic stress tolerance in maize: a random or planned process?, in *Proceedings of International Symposium on Plant Breeding August 17–22, 2003* (ed. A.R. Hallauer), Iowa State University Press, Mexico City, pp. 293–309.

51 Cassman, K.G. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 5952–5959.

52 Tollenaar, M. and Lee, E.A. (2002) *Field Crops Res.*, **75**, 161–169.

53 Duvick, D.N., Smith, J.S.C., and Cooper, M. (2004) *Plant Breed. Rev.*, **24**, 109–151.

54 Tinker, N.A. (2002) Why quantitative geneticists should care about bioinformatics, in *Quantitative Genetics, Genomics and Plant Breeding* (ed. M.S. Kang), CABI, Wallingford, pp. 33–44.

55 Barker, T., Campos, H., Cooper, M., *et al.* (2004) *Plant Breed. Rev.*, **25**, 173–253.

56 Edmeades, G.O., Bolaños, J., Elings, A., *et al.* (2000) The role and regulation of the anthesis-silking interval in maize, in *Physiology and Modeling Kernel Set in Maize* (eds M.E. Westgate and K.J. Boote), CSSA Special Publication No. 29, CSSA, Madison, WI, pp. 43–73.

57 Cahn, M.D., Zobel, R.W., and Bouldin, D.R. (1989) *Plant Soil*, **119**, 271–279.

58 Hund, A., Ruta, N., and Liedgens, M. (2009) *Plant Soil*, **318**, 311–325.

59 Campos, H., Cooper, A., Habben, J.E., *et al.* (2004) *Field Crops Res.*, **90**, 19–34.

60 Patterson, H.D. and Williams, E.R. (1976) *Biometrika*, **63**, 83–92.

61 Smith, A., Cullis, B., Luckett, D., *et al.* (2002) Exploring variety-environment data using random effects AMMI models with adjustments for spatial field trend: Part 2: applications, in *Quantitative Genetics, Genomics and Plant Breeding* (ed. M. Kang), CAB International, Wallingford, pp. 337–352.

62 Lebreton, C., Lazic-Jancic, V., Steed, A., *et al.* (1995) *J. Exp. Bot.*, **46**, 853–865.

63 Veldboom, L.R. and Lee, M. (1996) *Crop. Sci.*, **36**, 1310–1319.

64 Ribaut, J.M. and Hoisington, D. (1998) *Trends Plant Sci.*, **3**, 236–239.

65 Frova, C., Krajewski, P., Di-Fonzo, N., *et al.* (1999) *Theor. Appl. Genet.*, **99**, 280–288.

66 Tuberosa, R., Salvi, S., Sanguineti, M.C., *et al.* (2002) *Ann. Bot.*, **89**, 941–963.

67 Vollbrecht, E., Springer, P.S., Goh, L., *et al.* (2008) *Plant J.*, **54**, 888–898.

68 Ribaut, J.M., Hoisington, D., Banziger, M., *et al.* (2004) Genetic dissection of drought tolerance in maize: a case study, in *Physiology and Biotechnology Integration for Plant Breeding* (eds H.T. Nguyen and A. Blum), Marcel Dekker, New York, pp. 571–609.

69 Ribaut, J.M., Banziger, M., Betran, F.J., *et al.* (2002) Use of molecular markers in plant breeding: drought tolerance improvement in tropical maize, in *Quantitative Genetics, Genomics, and Plant Breeding* (ed. M.S. Kang), CABI, Wallingford, pp. 85–99.

70 Mugo, S. and Hoisington, D. (2001) Biotechnology for the improvement of maize for resource poor farmers: the CIMMYT approach, in *Enhancing the Contribution of Maize to Food Security in Ethiopia* (eds N. Mandefro, D. Tanner, and S. Twumasi-Afriyie), EARO and CIMMYT, Addis Ababa, Ethiopia, pp. 203–213 (Proceedings of the Second National Maize Workshop of Ethiopia, November 12–16, 2001).

71 Tsonev, S., Todorovska, E., Avramova, V., *et al.* (2009) *Biotechnol. Biotechnol. Eq.*, **23**, 1410–1413.

72 Prasanna, B.M. and Ribaut, J.M. (2005) Molecular approaches for improving maize productivity under abiotic stresses, in *Stresses on Maize in Tropics* (eds P.H. Zaidi and N.N. Singh) Directorate of Maize Research, New Delhi, India, pp. 462–472.

73 Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997) *Plant Physiol.*, **115**, 327–334.

74 Liu, Q., Kasuga, M., Sakuma, Y., *et al.* (1998) *Plant Cell*, **10**, 1391–1406.

75 Manuela, M.C., Maroco, J.P., Pereira, J.S., *et al.* (2003) *Funct. Plant Biol.*, **30**, 239–264.

76 Qin, F., Li, J., Zhang, G.Y., *et al.* (2003) *Acta Bot. Sin.*, **45**, 331–339.

77 Wang, Z.Y., Sun, X.F., Wang, F., *et al.* (2005) *J. Integr. Plant Biol.*, **47**, 873–880.

78 Giuliani, S., Sanguineti, M.C., Tuberosa, R., *et al.* (2005) *J. Exp. Bot.*, **56**, 3061–3070.

79 Thornsberry, J.M., Matsuoka, Y., Wilson, L.M., *et al.* (2001) *Nat. Genet*, **28**, 286–289.

80 Wilson, L.M., Whitt, S.R., Ibáñez, A.M., *et al.* (2004) *Plant Cell*, **16**, 2719–2733.

81 Comai, L., Young, K., Till, B.J., *et al.* (2004) *Plant J.*, **37**, 778–786.

82 Xu, Y., Skinner, D.J., Wu, H., *et al.* (2009) *Int. J. Plant Genomics*, **2009**, 957602.

83 Weil, C. and Monde, R. (2009) Tilling and point mutation detection, in *Handbook of Maize: Genetics and Genomics* (eds J.L. Bennetzen and S.C. Hake), Springer, London, pp. 585–596.

84 McCarty, D.R. and Meeley, R.B. (2009) Transposon resources for forward and reverse genetics in maize, in *Handbook of Maize: Genetics and Genomics* (eds J.L. Bennetzen and S.C. Hake), Springer, pp. 561–584.

85 Kanashiro, H., Calderon Vazquez, C.A., Ibarra Laclette, C., *et al.* (2008) Gene expression analysis and physiological responses of Mexican maize landraces under drought stress. 50th Annual Maize Genetics Conference, February 27–March 1, 2008, Washington, D.C., Abstract.

86 Jansen, R.C. and Nap, J.P. (2001) *Trends Genet.*, **17**, 388–391.

87 Hu, Y., Li, W.C., Xu, Y.Q., *et al.* (2009) *J. Appl. Genet.*, **50**, 213–223.

88 Wullschleger, S.D. and Difazio, S.P. (2003) *Comp. Funct. Genome*, **4**, 216–224.

89 Brunner, S., Fengler, K., Morgante, M., *et al.* (2005) *Plant Cell*, **17**, 343–360.

90 Candela, H. and Hake, S. (2008) *Nat. Rev. Genet.*, **9**, 192–203.

91 Arumuganathan, K. and Earle, E.D. (1999) *Plant Mol. Biol.*, **9**, 208–228

92 Schnable, P.S., Ware, D., Fulton, R.S., *et al.* (2009) *Science*, **326**, 1112–1115.

93 Wei, F., Zhang, J., Zhou, S., *et al.* (2009) *PLoS Genet.*, **5**, e1000715 (online).

94 Xu, Y. and Crouch, J.H. (2008) *Genomics of Tropical Crop Plants, Plant Genetics and*

Genomics: Crops and Models, vol. 1, pp. 333–370.
95 Bortiri, E., Jackson, D., and Hake, S. (2006) *Curr. Opin. Plant Biol.*, **9**, 164–171.
96 Shendure, J. and Ji, H. (2008) *Nat. Biotechnol.*, **26**, 35–45.
97 Kahvejian, A., Quackenbush, J., and Thompson, J.F. (2008) *Nat. Biotechnol.*, **26**, 25–33.
98 Metzker, M.L. (2010) *Nat. Rev. Genet.*, **11**, 31–46.
99 Paterson, A.H., Lan, T.H., Reischmann, K.P., *et al.* (1996) *Nat. Genet.*, **14**, 380–382.
100 Van Buuren, M., Salvi, S., Morgante, M., *et al.* (2002) *Plant Mol. Biol.*, **48**, 741–750.
101 Ware, D.H., Jaiswal, P., Ni, J., *et al.* (2002) *Plant Physiol.*, **130**, 1606–1613.
102 Liu, S., Zhang, X., Pumphrey, M.O., *et al.* (2006) *Funct. Integr. Genomics*, **6**, 83–89.
103 Chimenti, C.A., Marcantonio, M., and Hall, A.J. (2006) *Field Crops Res.*, **95**, 305–315.
104 Beck, E.H., Fettig, S., Knake, C., *et al.* (2007) *J. Biosci.*, **32**, 501–510.
105 Ozturk, Z.N., Talame, V., Deyholos, M., *et al.* (2002) *Plant Mol. Biol.*, **48**, 551–573.
106 Sharp, R.E., Poroyko, V., Hejlek, L.J., *et al.* (2004) *J. Exp. Bot.*, **55**, 2343–2351.
107 Nakazono, M., Qiu, F., Borsuk, L.A., *et al.* (2003) *Plant Cell*, **15**, 583–596.
108 Jeanneau, M., Gerentes, D., Foueillassar, X., *et al.* (2002) *Biochimie*, **84**, 1127–1135.
109 Grotewold, E. (2005) *Trends Plant Sci.*, **10**, 57–62.
110 Boyer, J.S. and Westgate, M.E. (2004) *J. Exp. Bot.*, **55**, 2385–2394.
111 McLaughlin, J.E. and Boyer, J.S. (2004) *Ann. Bot. (Lond.)*, **94**, 675–689.
112 Wang, W., Vinocur, B., Shoseyov, O., *et al.* (2004) *Trends Plant Sci.*, **9**, 244–252.
113 Pelleschi, S., Leonardi, A., Rocher, J.P., *et al.* (2006) *Mol. Breed.*, **17**, 21–39.
114 Wang, W., Vinocur, B., Shoseyov, O., *et al.* (2004) *Trends Plant Sci.*, **9**, 244–252.
115 Tran, L.S.P., Nakashima, K., Sakuma, Y., *et al.* (2004) *Plant Cell*, **16**, 2481–2498.
116 Capell, T. and Christou, P. (2004) *Curr. Opin. Biotechnol.*, **15**, 148–154.
117 Konstantinova, T., Parvanova, D., Atanassov, A., *et al.* (2002) *Plant Sci.*, **163**, 157–164.
118 Zhang, S.H., Peng, Z.B., and Li, X.H. (2000) *Sci. Agric. Sin.*, **33**, 34–39.
119 Smith, J.S.C., Duvick, D.N., Smith, O.S., *et al.* (2004) *Crop. Sci.*, **44**, 1935–1946.
120 Vinocur, B. and Altman, A. (2005) *Curr. Opin. Biotechnol.*, **16**, 123–132.
121 Ingram, J. and Bartels, D. (1996) *Annu. Rev. Plant Physiol.*, **47**, 377–403.
122 Song, F.B. and Wang, X.B. (2005) *Maize Physiology and Ecology Under Abiotic Stress*, Science Press, Beijing, pp. 10–120.
123 Wang, G.L. and Fang, H.J. (2002) *Plant Gene Engineering*, 2nd edn, Science Press, Beijing, pp. 61–73.
124 Shirasawa, K., Takabe, T., and Kishitani, S. (2006) *Ann. Bot.*, **98**, 565–571.
125 Saito, K., Hayano-Saito, Y., Maruyama-Funatsuki, W., *et al.* (2004) *Theor. Appl. Genet.*, **109**, 515–522.
126 Bajaj, S. and Mohanty, A. (2005) *Plant Biotechnol. J.*, **3**, 275–307.
127 Chen, T.H.H. and Murata, N. (2002) *Curr. Opin. Plant Biol.*, **5**, 250–257.
128 Tao, D., Yu, M., Feng-Ling, F., *et al.* (2008) *Biotechnology*, **7**, 258–265.
129 Quan, R., Shang, M., Zhang, H., *et al.* (2004) *Plant Biotechnol. J.*, **2**, 477–486.
130 Sakamoto, A. and Murata, N. (2002) *Plant Cell Environ.*, **25**, 163–171.
131 Tarczynski, M.C., Jensen, R.G., and Bohnert, H.J. (1993) *Science*, **259**, 508–510.
132 Abebe, T., Guenzi, A.C., Martin, B., *et al.* (2003) *Plant Physiol.*, **131**, 1748–1755.
133 Garg, N., Pundhir, S., Prakash, A., *et al.* (2008) *J. Comput. Sci. Syst. Biol.*, **1**, 021–040.
134 Sheveleva, E., Chmara, W., Bohnert, H.J., *et al.* (1997) *Plant Physiol.*, **115**, 1211–1219.
135 Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.J., *et al.* (1995) *Plant Physiol.*, **107**, 125–130.
136 Kishore, M.B.K., Hong, Z., Miao, G.H., *et al.* (1995) *Plant Physiol.*, **108**, 1387–1394.
137 Ramanjulu, S. and Bartels, D. (2002) *Plant Cell Environ.*, **25**, 141–151.
138 Roosens, N.H., Al Bitar, F., Loenders, K., *et al.* (2002) *Mol. Breed.*, **9**, 73–80.
139 Bartels, D. and Sunkar, R. (2005) *Crit. Rev. Plant Sci.*, **24**, 1–36.
140 Zhang, A., Jiang, M., Zhang, J., *et al.* (2006) *Plant Physiol.*, **141**, 475–487.

141 Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M. (2003) *Curr. Opin. Plant Biol.*, **6**, 410–417.
142 Gilmour, S.J., Fowler, S.G., and Thomashow, M.F. (2004) *Plant Mol. Biol.*, **54**, 767–781.
143 Nakashima, K., Kiyosue, T., Yamaguchi-Shinozaki, K., *et al.* (1997) *Plant J.*, **12**, 851–861.
144 Nelson, E.D., Repetti, P.P., Adams, T.R., *et al.* (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 16450–16455.
145 Mantovani, R. (1999) *Gene*, **239**, 15–27.
146 Shou, H., Bordallo, P., and Wang, K. (2004) *J. Exp. Bot.*, **55**, 1013–1019.

34
Barley: Omics Approaches for Abiotic Stress Tolerance

Nicola Pecchioni, Justyna Anna Milc, Marianna Pasquariello, and Enrico Francia

Abiotic stresses such as frost, drought, salinity, hypoxia, and mineral deficiency or toxicity frequently limit growth and productivity of temperate cereal crops, for which barley (*Hordeum vulgare* L. ssp. *vulgare*) could represent a model. Improving barley resistance to such constraints is thus fundamental in view of the expected climate change for minimizing the gap between potential and actual yield (the so-called "yield gap"), increasing the yield stability, and guaranteeing the sustainability of the crop. As different omics technologies have been developed during the past few decades, they enabled systematic analysis of changes that occur in plants in response to abiotic stresses. In this chapter, we focus on the "omics" contribution to the improvement of abiotic stress tolerance in barley. After a brief summary of the most relevant abiotic stresses that limit the crop yields worldwide, successful genomics approaches have been described, starting from the exploitation of germplasm resources. Structural and functional approaches that helped in understanding the mechanisms and the genetic bases of abiotic stress tolerance, when applied to barley and model species (mainly *Arabidopsis*, rice, and *Brachypodium*), have been reviewed as an important step toward crop tolerance improvement. Quantitative genetics and genetical genomics of abiotic stress tolerance have been discussed, as they represent both a huge source of information and a challenge for future holistic approaches. Then, we present an overview of the contribution of other omics sciences (e.g., proteomics, epigenomics, metabolomics, ionomics, and phenomics). In the last section, integrative (systems) biology, together with a series of strategies for the future, is proposed and discussed.

34.1
Abiotic Stresses Relevant for Barley

Throughout their domestication and breeding, man has driven the genetic improvement of cereals toward high yields in different environments. Notwithstanding, physical or abiotic stresses, particularly drought, low temperatures and salinity, impose major limitations on cereal productivity worldwide. Trying to synthesize

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some of its academic definitions, Levine [1] described "stress" as an adaptation syndrome to stressors, or stress stimuli; these disturb the homeostasis of an organism, which in turn, through a complex processing system, responds to the stress. In Levine's view, stress is then a complex concept consisting of three main subclasses, the input (stress stimuli), the processing systems, including the organism's subjective experience of stress, and the output (stress responses), with the basic difficulty that these subclasses interact. To quote Larcher, ". . .stress can be described as a state in which increasing demands made upon a plant lead to an initial destabilization of functions, followed by normalization and improved resistance. If the limits of tolerance are exceeded and the adaptive capacity is overtaxed, permanent damage or even death may result" [2]. According to Pahlich [3], a plant physiologist would consider how plants have to cope permanently with adverse environments, then stress is not an exception but rather a normal situation of plant life. However, for a plant breeder, the influence of stressors on the agricultural plant communities is one of the major known limits of their yield potential.

In the case of barley, *Hordeum vulgare* L. ssp. *vulgare*, abiotic stresses are responsible for significant grain yield losses on a global scale. Yet, under severe stress conditions, barley, as fourth world cereal in terms of harvested area, after wheat, rice, and maize, is one of the most important sources of energy for both humans and animals. Average world barley yield is of 2.8 ton ha^{-1}, according to FAO estimates (http://faostat.fao.org/), while its yield potential would be above 6.7 ton ha^{-1}, as measured for Argentina by Abeledo *et al.* [4]. The wide yield gap is significantly due to abiotic stresses, particularly drought and nitrogen, together with biotic ones, besides other technical and social limitations [4]. Globally, barley is cultivated in 54 129 438 ha (FAO's estimates for 2009), and its geographic distribution suggests how abiotic stresses have driven evolution, distribution, and ecology of both crop and whole genus *Hordeum*. In fact, the *Hordeum* species are diffused in temperate, subtropical, and arctic areas, from the sea level to heights of more than 4500 m a.s.l. in the Andes and Himalayas [5]. The same could be true for cultivated barley, widespread from the Northern boreal countries, as the last crop encountered before grassland toward the polar circle, up to the margins of the deserts [6].

From a physiological point of view, the different strategies adopted by plants to cope with stressful environments can be classified into the mechanisms of stress escape, avoidance, and tolerance [7]. The ability of barley to grow in very cold environments of cultivation is mainly an escape strategy: in the short summer of such climate regions only spring genotypes with a very short life cycle are grown. In totally different environments, the short growth cycle allows barley to set seed after rainfall winter growth, in dry and warm springs across the Mediterranean countries, when durum wheat also fails. Synchronizing life cycle to seasonal changes thus allows barley to escape extremely stressful conditions, although environmental adaptation also involves barley plant's ability to avoid or tolerate stresses such as frost, drought, or low soil fertility. From a breeding point of view, stress tolerance can be described as the capacity to maintain a constantly high yield, regardless of any environmental adversity (a concept known as yield stability).

A comprehensive classification of the abiotic (physical) stresses should comprise nutrients (nutrient imbalance); light (high light, UV, and darkness); water (deficit and flooding) and hypoxia; temperature (frost, chilling, and heat); salt; heavy metals and other chemicals; and mechanical (wind and touch). Among them, the most important and frequent stresses barley has to cope with are the following. Cold (frost) during winter, in the early vegetative phases, damages the fall-sown barley in the Northern Hemisphere. Drought and salt limit rain-fed barley yields in the Mediterranean, subtropical, and inner continental arid and semiarid areas of the world, from the vegetative to the grain filling phase, more often in the reproductive phase of its cycle. In the same areas, often barley experiences nutrient stress, particularly due to nitrogen deficiency. Cold stress in the reproductive phase is quite infrequent, except in certain regions of Australia where it is commonly due to cold winds [8]. Again in Australia, barley is exposed to toxic effects of imbalanced (excess) soil micronutrients such as boron [9]. In East Asia, when the barley crop is cultivated in paddy fields with a rice–barley double cropping system, it is often exposed to water damage and consequent soil hypoxia due to the Asian monsoon climate [5].

It is often underestimated that plants most often experience a combination of different abiotic stresses instead of a single physical stress. This is due to the complexity of the physical and biotic factors that affect and characterize natural and agricultural systems. For example, barley plants are exposed to heat and drought, drought and nutrients, salt and drought, rather than simply to drought. As a consequence, while generally abiotic stress "omics" experiments have often been performed by molecular biologists under controlled conditions (growth chambers, hydroponics, etc.), field experiments hold the greatest value for reality of the combined stress conditions. As it will be demonstrated in the chapter, a common part of the plant response/tolerance to multiple stresses surely exists. This was indirectly deduced by very similar cellular damages from different stresses. But it was also demonstrated, for example, by the improved tolerance to multiple abiotic stresses acquired by plants transformed by single key transcription factors such as *DREB1A* (dehydration responsive element binding 1A) [10] or *TaERF1* (*T. aestivum* ethylene-responsive factor 1) [11]. On the other hand, Mittler [12] correctly addressed the existence of specific responses to multiple stresses. He underlined how recent studies revealed that plants response to a combination of two different abiotic stresses has a unique component that cannot be directly deduced if each stress is applied individually. Notably, Rizhsky *et al.* [13] highlighted the plasticity of the genome of *Arabidopsis thaliana* (L.) Heynh. (hereafter, *Arabidopsis*), demonstrating its ability to respond to a combination of drought and heat stresses. Besides genes that were commonly or specifically induced by each single environmental factor, 454 transcripts were exclusively induced in plants exposed to both stresses simultaneously [13].

Moreover, it should be considered that there can be under natural conditions three kinds of recognized interactions between stresses: (i) no interaction, for example, between heat and chilling; (ii) positive interaction, for example, between light and ozone stresses; and (iii) negative interaction, for example, between

drought and nutrient. In other combined stresses, the kind of interaction is not known [12].

The cultivated and wild barley species within the *Hordeum* genus are part of the Triticeae tribe in the Poaceae family. Representing strategic main food and feed commodity crops in the Triticeae, wheats (bread and durum) and barley are among the First World crops in terms of cultivated areas and product quantities (with barley and wheat ranking sixth and ninth, respectively; http://faostat.fao.org/). Other cultivated members of the tribe of lower importance are rye, triticale, emmer, einkorn, and minor wheat species.

Among temperate cereal crops, it is well known that winter barley types are less tolerant to frost damage than winter wheat or winter rye and triticale. On the other hand, barley seems more adapted than wheat to shallow and poorly fertile soils or to arid environments [5]. Stanca *et al*. [5] and Walia *et al*. [14], on the basis of previous studies, clearly classify barley as more salt-tolerant than wheat, and as salt-tolerant member of the tribe. Barley (*H*. *vulgare* L.) could be considered a good model for the Triticeae among the temperate cereals, to study plant response to adverse environmental conditions. Its inbreeding behavior and diploidy make the genetic and genomic studies easier to perform compared to the polyploid wheats. The above cited wide range of adaptations, the availability of large genetic stocks, and the extended genome colinearity, not only with other members of the tribe but also with the genome of *Brachypodium distachyon* (L.) Beauv. (hereafter *Brachypodium*), are additional advantages for barley as a model [15, 16].

The "omics" approaches hitherto applied to understand the impact of environmental stressors on the final phenotype are here reviewed in the model diploid cereal barley, with the hope that they will help drive the "developmental trajectory" [1] of future cultivars to resilience under stress.

34.2
Genomics Approaches for Abiotic Stress Tolerance in Barley

Abiotic stresses influence plant growth and severely limit their yield potential, as already underlined. Although the genomes of Triticeae are large and still poorly characterized relatively to the model species, the diversity in the cultivated and wild gene pools for the Triticeae tells us that there is room for achieving greater tolerance. Research efforts have been and are mostly directed toward a better theoretical understanding of the genetic basis of the adaptive response of plants to stress. The complexity of abiotic stress responses, networks of signaling pathways, and interconnecting processes were dealt *in primis* with broad approaches of genomics coupled with bioinformatics [17, 18]. Therefore, the wide spectrum of genomics resources developed during the past two decades to facilitate the systematic analysis of the barley genome are reviewed here, with special emphasis to the best exploitation of theoretical knowledge for applied purposes. We are in fact convinced that the applied aspect of genomic research would deserve in coming years constantly increasing attention.

34.2.1
Genomics for the Exploitation of Barley Germplasm Resources

Plant genetic resources (PGRs) have been useful for both breeders and research community, at least since the first decades of the last century, for useful trait introgression. Plant germplasm collections respond today to the pressing need of preserving biodiversity, at its three recognizable levels. If, in fact, we would like to define the concept of biodiversity, we would better divide it into the three levels of intraspecific ("genetic"), interspecific, and (eco)system biodiversity (modified from Ref. [19]). This last one depends and causes at the same time a series of interactions between organisms, and between them and the physical stressors. From a conservation perspective, the intraspecific variation (in case of *H. vulgare* L.) can be more easily maintained in *ex situ* collections, both private and public, since its maintenance in the agricultural areas for a crop species cannot be pursued regardless of economic considerations. The interspecific one should be instead better preserved *in situ*; under such a condition, a natural dynamics of the diversity can, in fact, be maintained, in all the environmental stimuli, rather than in a "fixed" environmental situation like that of a germplasm collection. However, conservation *in situ* has the necessity of a land conservation project, and for the social and economic obstacles often encountered in such projects, crop relatives and other plant species are preserved in gene banks also at the interspecific level.

In the genomic era, the most valuable sum of the *ex situ* and *in situ* resources should be viewed at least as the primary (GP1) and secondary (GP2) gene pools. In the Harlan's concept, these two comprise (1) both cultivated and wild species easily crossable with the cultivated ones (GP1); (2) species that can be cross-bred only with difficulty with the cultivated one (GP2). In case of barley, GP1 is composed of *H. vulgare* L. ssp. *vulgare* cultivars and landraces, together with the wild *H. vulgare* L. ssp. *spontaneum* (K. Koch) Thell. accessions, while GP2 is composed only of the species *H. bulbosum* L. [20].

The greatest genomic value of the primary and secondary gene pools should be that of an immense "barley metagenome," boldly extending the proper concept of metagenome [21]. Such a value recently increased because of the advent of second-generation sequencing [22], and in the next decade could further increase with the availability of the third-generation sequencing technologies [23]. Because of its large genome, in the near future, at least the resequencing of the "gene space" of barley gene pools should allow "fishing" a huge reservoir of alleles for their transfer to the future barley cultivars. Apart from the technical limits described in Section 34.2.2, the association between allele variation and precise phenotypic variants should be taken into careful consideration as a bridle to the enthusiasm for genome resequencing. On the other hand, a bright development work and the use of prebreeding materials would be necessary. In fact, it has been demonstrated both in rice and in tomato how useful variation can be hidden in wild materials with lower phenotypic value [24].

Systematic collection of barley diversity on a worldwide scale started in the early twentieth century, after the Vavilov's theory and inputs [25]. Major collections (holding more than 10 000 accessions) are being conserved in Australia, Brazil,

Canada (Plant Gene Resources of Canada – PGRC), China (CAAS – Chinese Academy of Agricultural Sciences), Ethiopia, Germany (Leibniz Institute of Plant Genetics and Crop Plant Research – IPK), Japan (Okayama University), Mexico, Russian Federation (Vavilov Institute), South Korea, Sweden (Nordic Gene Bank), Syria (ICARDA – International Center for Agricultural Research in the Dry Areas), the United Kingdom, and the United States (USDA-ARS – United States Department of Agriculture, Agricultural Research Service). In the initial phase of development of the Global Strategy for the *Ex Situ* Conservation and Use of Barley Germplasm, ICARDA was commissioned by the Global Crop Diversity Trust, with major input from the IPK, Gatersleben, Germany, to coordinate the development of a global inventory of barley genetic resources in *ex situ* collections. It was demonstrated that the total of barley germplasm holdings at 47 major barley collections (those with more than 500 accessions) was of 402 000 accessions, and if 28 minor collections are also considered, the global total would rise to 405 000. Of the 290 820 accessions for which germplasm type is known, 15% are wild relatives, 44% are landraces, 17% are breeding materials, 9% are genetic stocks, and 15% are cultivars [26].

The Nordic Gene Bank *Hordeum* collection consists of 45 species and about 13 000 accessions. Furthermore, NGB stores many special collections, including the about 10 000 accessions of barley mutants, and the collection of near-isogenic lines (NILs) of wheat, oat, and barley with different resistance genes in a standard genetic background [27]. The barley genetic stock collection at USDA-ARS consists of over 2000 accessions collected worldwide. The accessions are entered as GSHO (genetic stock of *Hordeum*) on the GRIN (Germplasm Resources Information Network) database. Also remarkable is the public availability for the research community of the Oregon Wolfe Barley (OWB) population of 94 doubled haploids (DHs) [28] that were derived from the F1 of a cross between the dominant and the recessive parental marker stocks developed in 1972 at Brandon, Canada, by R.I. Wolfe. Ninety-four Oregon Wolfe Barley Genetic Stocks are also included in the GRIN database (GSHO 3325–3418). The European Barley DataBase (EBDB, http://barley.ipk-gatersleben.de/ebdb) is a central activity of the Barley Working Group and includes about 155 000 accessions from 23 European countries and about 38 000 additional accessions from three non-European gene banks, Australia (the Australian Winter Cereals Collection – AWCC), Syria (ICARDA), and Japan (Barley Germplasm Center). Moreover, more than 1000 accessions from the International Barley Core Collection (BCC) are documented [29]. Such a collection, which contains both wild and cultivated genotypes, was developed thanks to an international coordinated effort [30], and could be valuable for allele mining.

A database on barley genes and genetic stocks (BGS, http://ace.untamo.net/) was also developed using AceDB and the data model is similar to the one used in GrainGenes. This server contains information on morphological barley mutants and their genetic background. Hyperlinks to some gene banks holding the mutant accessions and parents (ARS/GRIN/NSBC or NGB) have been added to facilitate easier access to stock material containing specific alleles.

From a phenotypic point of view, diversity for abiotic stress tolerance can be found in various genetic stocks [5]. For example, Nevo [31] reported that the root system of

the barley direct ancestor *H. vulgare* L. ssp. *spontaneum* penetrates deeply into the warm steppes and deserts. A large number of Asian cultivated barleys were tested for tolerance to hypoxia in terms of water sensitivity, pregermination and postgermination flooding. From the 1980s, a great number of barley accessions have also been evaluated for salt tolerance in different experiments and under different stress conditions [5]. From one of such *H. vulgare* L. ssp. *spontaneum* accessions, CPI-71284-48, the "wild" allele of the *HvNax3* gene reducing shoot Na^+ accumulation is going to be cloned by colinearity analysis with rice and *Brachypodium* [32].

For any project involving allele diversity mining, two approaches are possible. The former is based on sequencing, while the latter is based on EcoTilling. In both cases, at least for the moment, the experiments have been driven by trait – and underlying genes – priority [33]. Moreover, since the "true" allele mining should include the coding, noncoding, and regulatory regions of a gene, the availability of full genomic sequences of the target genes should be available. To realize the potential of allele mining in genetic resources, some of the international research institutes maintaining crop germplasm collections have initiated studies to characterize the allelic diversity of crop plants. In perspective, although many hurdles still need to be overcome, the success of allele mining strategies is expected to result in a real quantum leap in the use of PGRs. At present, however, allele mining seems to be still a quite underexploited approach to unlock the diversity in the collections of the world gene banks [34]. The few examples of allele mining available in literature for barley mostly focused on cultivated germplasm. These efforts were concerned with few genes, mostly amylase and others not involved in abiotic stress response, apart from the allele survey of the couple *VRNH-1/VRN-H2*, regulating the vernalization requirement and partially involved in frost stress tolerance, in 429 spring, facultative, and winter European varieties [33, 35].

One challenge for allele mining in barley PGRs, as well as in other species, is an appropriate selection of genotypes. In fact, screening the entire collection would be helpful to find rare alleles, for sure, but this would be an enormous task for a research institute. Hence, a prioritization of genotypes should be done, like the development of core or even minicore collections [36], as the BCC, which could constitute the starting materials for the mining efforts. Moreover, as already underlined, accurate phenotyping methods are needed to increase the efficiency of allele mining. Finally, efficient computational tools are necessary to ensure the access to useful alleles [37]. Other issues that should be solved are then the demarcation and characterization of putative promoter regions and the reduction of the cost per data point when analyzing allele sequence variants.

34.2.2
Barley Structural Genomics Resources

Barley "H" genome, as those of the other cultivated Triticeae, is a large genome of >5000 Mb, according to Bennett and Smith [38]. However, we would like to reinforce the concept of barley as a genomic model for wheats, for the reasons discussed in Section 34.2.1, and because barley science provided experimental results that

assumed a general importance for understanding the Poaceae and plant biology [16]. In the time space between the end of the past century and the first decade of the twenty-first century, which has seen the first plant small- and medium-sized genomes fully sequenced, it has partially closed the gap between the size of large genomes, such as barley and wheat, and the technological feasibility of sequencing them at reasonable costs, through next-generation sequencing (NGS) [22]. Although so far it is not definitely demonstrated that large genomes like barley that feature >80% of repetitive DNA can be fully sequenced, recent research efforts created new barley genomic resources that bring the goal closer [16]. It was thus shown that NGS technologies can be suitable to determine gene content of barley BAC clones [39], can support automated repeat annotation of barley genomic DNA [40], can lead to correctly assembled BAC clone *de novo* sequences [41], and can allow by whole-genome shotgun (WGS) the characterization of the composition of the barley genome [42]. These first glances at the composition of the barley genome promise future advancements toward understanding of barley biology, including that of abiotic stress response. The availability of a fully ordered reference genome sequence could, in fact, provide a physical "harbor" to link in a more coherent network the other genetics and omics discoveries about abiotic stress biology. The steps and resources that led to the present situation of the barley structural genomics, particularly those relevant to abiotic stress response, are reviewed here.

Resources for barley structural genomics have improved over the past two decades, initiated in 1991 with the release of the first barley genetic map [43]. Now, about 70 barley maps with a large number of molecular markers are available, and a comprehensive overview of published genetic map resources in barley can be obtained from the GrainGenes database at http://wheat.pw.usda.gov/ggpages/map_summary.html. In recent years, there has been a significant progress in both marker density and convergence, leading to consensus maps for the species. Consensus maps containing 1230 markers (RFLP, AFLP, SSR, and SNP) from three doubled haploid populations [44] and 3458 markers (RFLP, AFLP, and SSR) from six mapping populations [45] were recently developed. Varshney *et al.* [46] produced a 775 SSR consensus map by joining six independent maps. The latest technological developments in barley genomics and genetics include the Diversity Arrays Technology (DArT), which allowed for the simultaneous analysis of several hundred gene loci and resulted in a high-density map of barley molecular markers linked to agricultural traits [47]. Wenzl *et al.* [48] created a consensus map containing 2935 markers combining DArT with RFLP, SSR, and STS from nine mapping populations. Hearnden *et al.* [49] combined 1000 SSR and DArT markers on a map from a wide cross. Potokina *et al.* [50] combined SNP and other transcript-derived markers to position 1596 loci on the "Steptoe" × "Morex" linkage map.

Another step forward from genetic maps to genome knowledge was taken thanks to expressed sequence tag (EST) sequencing, later with EST assembling, and particularly with their anchoring to genomic positions. The first initiative of systematic EST sequencing to establish a public EST database on wheat and barley has

been the ITEC (International Triticeae EST Cooperative), proposed at the Ninth International Wheat Genetics Symposium held at Saskatoon, Saskatchewan, in August 1998 (http://avena.pw.usda.gov/genome/). ITEC, starting with only six wheat ESTs and a handful of other Triticeae ESTs on record in 1998, was the first to produce thousands of barley ESTs, among others, from a cold-induced leaf cDNA library [51]. Later, Zhang *et al.* [52] generated more than 100 000 barley ESTs from 22 cDNA libraries representing tissues at various developmental stages, and with this began large-scale sequencing programs for the development of ESTs from various cDNA libraries. Today, progress made in the past 12 years has resulted in the generation of 502 620 ESTs for *H. vulgare* ssp. *vulgar*e covering different cDNA libraries from various stages of plant development and tissues, challenged with abiotic and biotic stresses (http://ace.untamo.net/ release 100110, October 1, 2010). The alignment of these ESTs led in the past decade to the identification of 43 306 tentative consensus (TC) sequences and 39 502 singletons (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=barley, Release 11.0, April 14, 2010). Searching the annotations with the abiotic stress-related keywords, one can find 11 entries for "drought," 9 for "water stress," 74 for "salt," 123 for "cold," and 37 for "heavy metal."

At the same time, a broad range of online bioinformatic tools and databases have been developed. These, such as Gramene (http://www.gramene.org), GrainGenes (http://wheat.pw.usda.gov), PlantGDB (http://www.plantgdb.org), and HarvEST (http://harvest.ucr.edu), help scientists to formulate and test their genomic hypotheses and to share their results with the barley research community, through the publication of genetic loci where the ESTs have been mapped thanks to different efforts. In fact, on the one hand the *in silico* expression studies on the EST data sets [52] provided snapshots of the transcriptome of diverse plant tissues at different stages, aiding in gene discovery, and allowed identification of metabolic and regulatory networks [18, 53]. On the other hand, several mapping projects were targeted to establish EST-based molecular maps [44, 49, 54, 55]. Among the first maps, the functional genetic map of Tondelli *et al.* [54] was based on three different mapping populations and on regulatory candidate genes (CGs) for abiotic stress tolerance; an integrated barley transcript map comprising 1000 EST-based markers and 200 anchor markers from previously published data was generated by mapping them in three DH populations, as a tool for the integration of genetic and physical mapping information [55]. Recently, nearly 3000 ESTs were mapped on a high-resolution transcript linkage map of barley created using a single DH mapping population and only PCR-based assays [56]. A dense consensus map based on transcribed gene SNPs derived from four mapping populations was also developed by utilizing the Illumina GoldenGate assay (Illumina Inc., San Diego, CA) [57], and Schulte *et al.* [16] estimated that the number of nonredundant barley genes mapped so far reached more than 5000. Eventually, in the near future the strategy used in the small genome species rice could be adopted for barley, where the available reference genome sequence allowed a high-throughput genetic mapping, on a gene-by-gene basis, by whole-genome sequencing of a segregating population [58].

At present, the high-density molecular maps based on gene-derived markers constitute the genetic scaffold for barley physical genome mapping. During the past years, core public resources have been established by generating bacterial artificial chromosome (BAC) libraries [59]. Libraries from cultivar "Morex" [60], "Cebada Capa" [61], and "Haruna Nijo" [62] have been reported, with several others already constructed and being in the making. The BAC libraries represent a useful tool not only for physical map construction but also for isolating several genes of agronomic interest by positional cloning. As one of the last examples of such use for abiotic stress tolerance, Shi *et al*. [63] developed a BAC library of the barley doubled haploid line "CS134," which contained tolerance alleles from the genotype "Sahara" at four boron tolerance loci, and one of the BAC clones facilitated isolation of the boron tolerance gene *BOT1* at its locus on chromosome 4H. As already outlined, EST dense maps can function as a framework for physical mapping of barley. One of the first efforts in physical mappping of the barley genome concerned the assignment of physical positions to the mapped ESTs, to chromosome arms, by using cytogenetic stocks, such as barley–wheat addition lines [64]. Such low-resolution physical mapping approach is a useful first step in developing BAC contigs for genome sequencing. However, better estimates of physical arrangements of BAC clones would be necessary in case of an ordered sequencing strategy. In the past years, increasing evidence has been found that genes are not randomly distributed across the barley genome, but are confined to a "gene space," which mainly covers the distal parts of the chromosomes [59]. Experimental evidences for the existence of a gene space has been gained from screening a barley BAC library with EST-derived probes, which showed a significant nonrandom distribution across the BAC clones [65]. The existence of a gene space also enables the researchers to focus analysis on gene-rich regions. By following an NGS sequencing strategy, Wicker *et al*. [39] were able to identify gene-containing "Morex" BACs by 454 sequencing. Some time later, by an alternative strategy, through the screening of the "Morex" BAC library using EST-derived, pooled "overgo" probes, Madishetty *et al*. [66] also identified the gene-containing barley BACs. Hybridization using "overgo" probes is an established approach for screening arrayed BAC libraries, which consists in annealing two 24-base or 22-base oligonucleotides with an 8-bp overlapping region and filling in the overhanging bases with Klenow enzyme and radiolabeled nucleotides. The probes that result from this procedure can be multiplexed and are characterized by low background hybridization; Madishetty *et al*. [66] have improved the yield of "overgo" positive clones using reduced levels of radioisotopes and enzyme.

The identified gene-rich BACs have been selected from cultivar "Morex" to initiate sequencing of all gene-containing regions of the barley genome by an international effort, coordinated through the International Barley Sequencing Consortium (IBSC, http://barleygenome.org). The international consortium was composed of eight founding institutions from six countries and was established in 2006; it was joined by China in 2010. Owing to the cost and technical difficulties, an immediate start could not be made with sequencing of the entire large complex genome of barley, as explained by its high content of repetitive DNA [16]. Therefore, a stepwise approach

was chosen that included the preparation of the necessary genomics and bioinformatics tools. The envisaged stepwise procedure should combine the strengths of map-based sequencing of a minimal tiling path with paired-end WGS sequencing [16]. To test the feasibility of the NGS methods for *de novo* sequencing, Wicker *et al.* [39] used 454 technology to sequence four barley BAC clones and compared the results with those from ABI Sanger sequencing. All gene-containing regions were covered efficiently and with high quality. Then, a pool of barcoded barley BACs was sequenced using the GS FLX platform and assembled correctly. It was shown that the gene-containing regions seem to assemble completely and uninterruptedly, thus making this approach suitable for detecting complete and positionally anchored genes [41]. The building of a genome-wide physical map of barley genome (*H. vulgare* L. ssp. *vulgare* cv. "Morex") is based on high information content fingerprinting (HICF) of BAC clones that are provided from five different BAC libraries (2x *Hind*III, *Eco*RI, *Mbo*I, and random sheared [67]). Cultivar "Morex" had been chosen as a genomic reference because numerous resources have already been generated using this genetic background. The complete physical map will comprise a 13–14-fold haploid genome coverage. In order to anchor the BAC contigs to the genetic map of barley, so far more than 6000 marker/BAC relationships were established. Anchoring is performed by PCR- and/or hybridization-based screening of BAC libraries with gene-based and genomic DNA-based marker types [68]. An IBSC goal is to obtain 10 000 mapped barley genes anchored, and high anchoring throughput can be obtained by means of available multiplex marker platforms as DArT and the Golden Gate assay [16]. In parallel, using low-pass shotgun sequencing, Wicker *et al.* [40] generated more than 500 Mb of Illumina/Solexa sequences from barley total genomic DNA, representing about 10% of genome equivalent. Then, to obtain a whole-genome sequence sample, two runs of 454 (GS20) snapshot sequencing on genomic DNA of barley cv. "Morex" were performed by Wicker *et al.* [42], which yielded approximately 1% of a haploid genome equivalent. Almost 60% of the sequences turned out to represent known transposable element (TE) families, while another 9% represented novel repetitive sequences. IBSC initiated the sequencing of the barley genome in 2006, but the time for its completion is hard to estimate (minutes of the IBSC Business Meeting at Plant Animal Genome XVIII, January 2010; http://barleygenome.org).

34.2.3
Role of Model Species

Many recent advances in genomics research have been based on model species. In the case of grasses, although the feasibility of sequencing large genomes like that of barley has been demonstrated, the main role of the model grass genomes of rice and false brome (*Brachypodium*) is in the synteny-based genomic research. Recently, increased genomic data for cereals allowed Bolot *et al.* [69] to propose a model for the evolution of the grass genomes from a common ancestor. On the basis of their data, an "inner circle" comprising five ancestral chromosomes was defined providing a new reference for the grass chromosomes and new insight into their ancestral

relationships and origin. In barley, Mayer *et al*. [70], through comparison against the reference genomes of rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*), and against wheat (*Triticum aestivum*) and barley expressed sequence tag data sets, estimated the presence of 4600–5800 genes on chromosome 1H. They identified conserved gene content between chromosome 1H, known syntenic regions of both rice chromosomes 5 and 10, and sorghum 1 and 9. Informed by the syntenic relationships between the two reference genomes, genic barley sequence reads were integrated and ordered to deduce a virtual gene map of barley chromosome 1H. Recently, a collinear structure, although with variations in the number of *CBF* elements (14 in barley, 9 in *Brachypodium*, and 3 in rice), was described between the *FR-H2* frost tolerance locus of barley and the corresponding *CBF* clusters of rice and *Brachypodium* [71].

Besides the synteny-based genomic studies, the model species *Brachypodium* deserves a particular interest for the abiotic stress biology since it is a temperate wild grass species [72], evolved in an ecological situation very similar to that of wheat and barley and therefore most likely exposed to a very similar array of stimuli. Its small genome size has been entirely sequenced; Sanger sequencing was used to generate 9.4× coverage and the final assembly of 83 scaffolds covers 271.9 Mb. Sequence scaffolds were aligned to a genetic map to create pseudo-molecules covering each chromosome [73]. Moreover, the false brome species presents self-fertility, a short life cycle, diploidy in the ecotypes, the subject of genomic studies, and simple growth requirements [74]. Genetic and genomic research resources for *Brachypodium* have evolved with the development of diploid inbred lines [75] and the establishment of a high-efficiency transformation system [76]. Genes most suitable for normalizing the gene expression data in *Brachypodium* have been identified [77]. Diverse collections of *Brachypodium* accessions are valuable resources for examining the natural variation of important traits such as its tolerance to abiotic stresses. The wide phenotypic variation in *Brachypodium* in response to drought stress has been recently explored and can be used to identify genes and alleles for the complex trait of drought tolerance [78].

34.2.4
Barley Functional Genomics Approaches for Abiotic Stress Tolerance

A major aim of functional genomics is to understand on a genome scale the metabolic and transcriptional networks within the structural and functional contexts of cells, tissues, and organs in a time course. Two approaches can be distinguished for studying the function of "stress genes." The first "functional" approach, considers stress genes as those crucial for response to a given stress, implying that mutational gain or loss of function of genes involved should result in altered stress tolerance. The second, the "inducible" approach, considers stress genes as those induced under the stress conditions and is based on the study of their modulation under stress, through differential screenings and displays and microarray methodologies [79].

In case of the functional genomics of barley's response to abiotic stresses, the most investigated field has been the "inducible" one, with a considerable amount of transcriptomic data generated. In comparison, less information derived from the

functional approach, as well as for the not-easy technical route that barley transformation takes. However, significant functional genomic resources such as TILLING (targeting-induced local lesions in genomes) populations have been produced, and these might be valuable tools for demonstration of hypotheses about the mechanisms of tolerance.

34.2.4.1 **Reverse Genetics Platforms**

To aid the "functional approach" analysis, efficient transformation systems and genome-wide systems for reverse genetics were developed for barley, including transgenic insertional mutagenesis and nontransgenic platforms such as TILLING [59]. All these systems were transferred to barley a few years after their initial development as mature technologies in *Arabidopsis* [80].

As far as transgenic insertional mutagenesis is concerned, the initial necessary step was the establishment of an efficient transformation system. The first studies of successful barley transformation in the early 1990s used biolistic techniques, followed after few years by *Agrobacterium*-based techniques to introduce DNA to immature embryos; these remain the target tissue of choice for obtaining high transformation efficiencies. *Agrobacterium*-mediated transformation of barley has now become more efficient in recent years with the development of new methods and protocols making high-throughput experiments more achievable [81]. As an alternative, *Agrobacterium*-based transformation system that uses microspore cultures as the target tissue was developed [82]; using this approach, homozygous doubled haploid plants can be obtained at high frequency through chromosome doubling. The successful introduction of Ac/Ds elements to knockout gene functions [83, 84] was the beginning of the establishment of gene tagging functional genomics in barley. The two-component maize transposon system *Ac/Ds (Activator/Dissociator)* is a widely used system for transposon-based insertional mutagenesis in plants, exported from maize to other species. In a heterologous two-element system, the nonautonomous Ds element may be activated by crossing the Ds-containing plant with an individual carrying the transposase gene (AcTPase) [85].

After the first results of Koprek *et al.* [83] and Scholtz *et al.* [84], insertional mutagenesis approaches in barley were thus used in the last decade to create both gain-of-function [86] and loss-of-function [87, 88] mutant platforms. The former approach, the last one achieved in the species in order of development, involves a random genomic insertion of either promoter or enhancer sequences that produce novel, dominant mutations by overexpression of endogenous genes (activation tagging). As regard activation tagging in barley, Ayliffe *et al.* [86] developed a tagging system where a modified Ds element containing two maize polyubiquitin promoters (UbiDS) was utilized for activation of transcription of adjacent flanking sequences. This study demonstrated the feasibility of activation tagging in barley, an approach particularly useful in large cereal genomes where gene redundancy may preclude gene identification through recessive loss-of-function mutations.

The systematic development of transgenic barleys overexpressing candidate genes for abiotic stress response could also have, other than an applied value, a theoretical

value, for validation of their functions in relation to abiotic stress tolerance. The possibilities for increasing tolerance to abiotic stresses by genetic transformation with candidate genes are enormous, while it is notable that the actual production of transgenic plants with demonstrably improved abiotic stress tolerance had been slow and limited to few species [89, 90]. Still there are only a few reported applications of transgenic barley for improving tolerance to abiotic stresses. Very recently, Morran *et al.* [91] generated transgenic wheat and barley plants expressing the TaDREB2 and TaDREB3 *T. aestivum* DREB transcription factors, with both constitutive (double 35S) and drought stress-inducible (ZmRab17) promoters. They obtained resistance to both drought and frost stresses, and the induction of other *CBF/DREB* genes, together with a large number of stress-responsive *LEA/COR/DHN* (late-embryogenesis abundant/cold-regulated/dehydrin) genes. Transgenic barley plants expressing *ALMT1* (Al-activated Malate Transporter 1) gene of wheat that encodes a malate transporter associated with malate efflux and aluminum (Al) tolerance were developed [92]. The results demonstrated that *ALMT1* was able to confer tolerance to Al in acidic soils. Barley genes had been more widely used to improve stress tolerance once introduced in other transgenic plants. For instance, expression in rice of the barley *HvHVA1*, a LEA gene, was one of the first reports of genes conferring tolerance to salt stress in plants [93]. The same transgene allowed rice to stand water deficit [93, 94], while transgenic overexpression of *HvCBF4* in rice resulted in an increase in tolerance to drought, high salinity, and low temperature without stunting growth [95].

A limit of the use of transgenesis to systematically modulate in barley the expression of the inserted genes is intrinsic in the methodology; that is, the reported experiments are based on hypotheses of action for single genes. Moreover, in the barley private breeding sector, a high-throughput transformation system similar to the "FAST Corn" system of maize (http://www.pioneer.com) does not exist. The technology consists in introducing large numbers of transgene combinations into corn quickly, potentially thousands per year, and using automated handling and precise digital imaging technologies to assess how specific transgenes affect a plant throughout its life cycle. The Pioneer maize seed company used the FAST Corn technology in the recent years, with which drought-tolerant transgenic hybrids were developed (DroughtII program, http://www.pioneer.com). However, a transient assay system based on bombarded leaf epidermis was developed and proved to be useful, to directly assess gene function in barley and wheat suffering from powdery mildew, a biotic stress caused by the fungus *Blumeria graminis* [96, 97]. This system, which can be used not only for transient overexpression of genes but also for transient-induced gene silencing (TIGS), has been further developed using GATEWAY technology in order to enhance throughput [98]. It is worth mentioning such a system of reverse genetics since a number of barley mRNAs homologous to drought response genes were selected and targeted by TIGS in microprojectile-bombarded barley leaves showing the value of the TIGS system for functional prescreening of a larger number of dehydration stress-related candidate genes [99].

As regard to the generation of loss-of-function mutants for barley functional genomics, a system for targeted gene tagging and local saturation mutagenesis based

on maize transposable elements (Ac/Ds) was reported [88]. With a long-term objective to develop and make available to the research community a comprehensive set of Ds insertion lines in barley containing a single Ds element assigned to a genomic position, on a barley linkage map, two complementary approaches were applied. In the first approach, a set of Ds-inserted lines was created by reactivating few original single-copy Ds plants by crossing them with AcTPase expressing plants and then recovering the transposed lines. Because it is known that Ds element tends to transpose at genomic positions close to the original Ds locus, to increase the number of genomic regions where it would have been possible to inactivate genes by local transposition, a second set of Ds-inserted lines was created. These lines were obtained by introducing Ds-carrying T-DNAs via *Agrobacterium* transformation. Single Ds insertions were then mapped genetically to verify the chromosome coverage. Sequence analysis of flanking regions for transposed Ds and T-DNA inserted Ds revealed a predominant insertion into nonredundant, gene-containing regions of the barley genome. Moreover, the observed high transposition frequencies demonstrated that the Ds launch pads could provide a solid base for functional genomics in barley [88]. In their study, Singh *et al*. [87] generated 100 multigenerational barley Ds insertion lines since no previously published data had been available in barley or wheat regarding the capability for consecutive Ds remobilization during generation advance. It was demonstrated that observed frequencies of reactivation of primary, secondary, tertiary, and quaternary TNPs (transposants–transposons insertion lines) could be sufficient for a saturation mutagenesis approach using Ds.

A gene trap system based on Ac/Ds was reported in barley as a valuable tool for knockout mutation, as well as forward and reverse genetic screens [100]. Gene trapping is a high-throughput insertional mutagenesis approach, in which a gene-trapping cassette consisting of a reporter gene and/or selectable genetic marker is used. The gene traps are characterized by splice acceptor sites and sometimes by an intron upstream of the reporter gene coding region. Such a structure facilitates the expression of in-frame reporter protein fusions regardless of insertion into intron or exon sequences. Until then, gene trap and enhancer trap approaches in monocots had exclusively been reported in rice; Lazarow and Lutticke [100] thus expanded the number of genomics tools available to the barley research community. The frequent transposition of the gene trap construct and the observed efficient expression of the reporter gene demonstrated that this approach could represent a significant step toward large-scale gene trapping in the crop.

Although insertional mutagenesis is a powerful, much more scalable tool for generating novel mutants, it has some limitations. These include the impossibility of studying the function of multigene families, the difficulty to reach genome saturation, and the possible parallel disruption of several genes due to the multiple insertions [85, 101]. These obstacles can be overcome by posttranscriptional gene silencing (PTGS), an RNA-mediated systemic silencing mechanism that permits to specifically silence or knock down the expression of targeted gene in plants. The discovery of the RNA interference (RNAi), in which double-stranded RNAs (dsRNA) trigger degradation of a target mRNA containing homologous sequence [102], led to the development of different and efficient dsRNA-mediated gene silencing

methods [85]. In plants, a particularly effective method for induction of dsRNA-silencing is transformation with a chimeric gene construct producing hairpin RNA (hpRNA). Such construct consists of inverted regions of the target gene sequence, separated by an appropriate spacer, which were shown to activate RNAi pathways in almost 100% of the transformed plants [103]. In a study by Wang *et al.* [104], in which plants were transformed in an attempt to create transgenic barley protected against the pathogen BYDV (barley yellow dwarf virus), out of 25 independent barley lines transformed with the BYDV-PAV hpRNA construct, 9 showed extreme resistance to the virus, and the majority of these contained a single transgene. However, hpRNA technique cannot be applied to genes whose silencing may block plant regeneration or result in embryo lethality. To solve these problems, Guo *et al.* [105] developed a chemical-inducible Cre/loxP (CLX) recombination system to trigger conditionally RNAi in plants, by the expression of an intron-containing inverted repeat RNA. They showed that it can be used to induce silencing of both transgenes and endogenous genes at different developmental stages, at high efficiency, and without any detectable secondary effects. The Chimeric REpressor gene-Silencing Technology (CRES-T) system was developed as a novel method for gene silencing, in which a transcription factor, which was converted into a strong repressor by fusion with the SRDX repression domain, suppresses the expression of target genes overcoming endogenous and functionally redundant transcription factors. In this way, dominant negative phenotype could be induced in genes causing agronomically desirable traits [106]. Repression of IDEF2, a novel transcription factor of rice and barley, which specifically binds to the iron deficiency-responsive *cis*-acting element 2 (IDEF2), obtained by the RNAi technique and CRES-T, was reported to cause aberrant iron homeostasis in rice [107]. A recent example of RNAi technique applied to study plant response to abiotic stress is an RNAi-induced CBF depletion in the Versailles core collection of *Arabidopsis*, characterized by strong variability in *CBF* gene expression after a cold exposure [108]. RNAi lines targeted against three *CBF* genes were produced in eight different accessions to examine the quantitative contribution of *CBF* expression to plant freezing tolerance. Analysis indicated a tight coregulation between *CBF1* and *CBF3* expression levels, while expression of *CBF2* seemed to be regulated in an independent way. The analysis of freezing tolerance of the RNAi lines also showed clear variation in the effect of downregulation of *CBF* expression among natural accessions.

Virus-induced gene silencing (VIGS) strategies have proven very useful in the analysis of gene function in dicot plants, but still only few demonstrations of effective VIGS in monocots have been reported. These include some examples of studies in barley, although not related to abiotic stress response, where barley stripe mosaic virus (BSMV) – the most effectively used virus in monocots for VIGS [109] – was used as a vector for silencing of barley phytoene desaturase (*PDS*) gene [110], for silencing of barley P23k involved in secondary wall formation [111], and finally for silencing of genes required for resistance toward *B. graminis* [112].

Targeting-induced local lesions in genomes was developed as a reverse genetics strategy that provides an allelic series of induced point mutations in genes of interest. This strategy, which allows rapid mutational screening to discover induced lesions,

has different advantages over other methods, as it does not require transformation or RNAi techniques and it can be applied to many crops. In barley, a TILLING resource has been generated using ethylmethanesulphonate (EMS) chemical mutagenesis of "Optic," a European two-row malting variety [113]. A few years later, an EMS TILLING population comprising 10 279 M2 mutants in the two-rowed malting cultivar "Barke" was developed at IPK Gatersleben [114], which has been used in the generation of other genomic resources in barley (~150 000 ESTs, DH mapping population). The TILLMore [115] population is the third, sodium azide-mutagenized TILLING collection of 5 000 M3 mutants of the cultivar "Morex", which is available at the University of Bologna [116]. A mutagenized population of about 10 000 lines (M3) has been recently developed in the Danish barley variety "Lux" by means of sodium azide. The population was used for detection of induced point mutations in two dehydrin genes *DHN12* and *DHN13*, as a proof-of-concept of the usefulness of such a TILLING resource for high-throughput mutation discovery [117].

34.2.4.2 **Transcriptional Profiling**

The transcriptome is by definition a set of all transcripts in one or a population of cells at a specific developmental stage or physiological condition. With present technologies, it allows to comprehensively profile all the information provided by the complete set of RNA transcripts (mRNAs, noncoding RNAs, and small RNAs), to understand gene structure including splicing patterns and other posttranscriptional modifications. It enables, therefore, to study the changes in barley gene expression under abiotic stress conditions. Diverse technologies have been developed to study the transcriptome, following hybridization- and sequence-based approaches; as is well known, the former consists in the hybridization of retrotranscribed RNAs with custom-made or commercial high-density (oligo) microarrays, while the sequence-based approaches directly determine the transcribed nucleotide sequences (for a review see Refs [118, 119]).

34.2.4.2.1 **Available Platforms** The availability of barley EST resources led to the development of the first generation of platforms for transcriptome analysis, from cDNA-based to oligonucleotide-based Affymetrix and Agilent microarrays. The Affymetrix 22 K Barley1 GeneChip [120] represented approximately 20 000 barley unigenes; annotation information for the Barley1 array is hosted on the NetAffx Analysis center at Affymetrix company. Moreover, HarvEST is functioning as an EST database-viewing software in support of the Affymetrix Barley1 GeneChip, enabling probe set annotation export, graphical displays of probes on unigenes, and other "Search the Barley Chip" functions. HarvEST Barley, Version 1.77 (January 9, 2010), has several additional features: barley genetic map viewer, including rice and *Brachypodium* synteny view, "Morex" BACs anchored to nearly 50% of the 2943 mapped SNP loci, and other few more. Barley Gene Expression Array on the Agilent Platform was developed in a 4 × 44 K slide format, using Agilent's 60-mer SurePrint technology. It has a comprehensive transcriptome coverage with 43 803 barley probes represented and content sourced from RefSeq, UniGene, TIGR Transcript Assemblies, and TIGR Gene Indices (http://www.genomics.agilent.com).

The increasing number of studies on plant transcriptomes, mainly in the past decade, has led to an accumulation of profiling data; and this in turn led the scientific community to provide itself with Web-based searchable databases to store the wealth of transcriptomic data. On the top of the already cited HarvEST bioinformatic tool, the online Plant Expression Database (PLEXdb), previously known as BarleyBase (http://www.plexdb.org), has been created to store, visualize, and statistically analyze Barley1 GeneChip data [121]. It provides public access to experiments done with GeneChip hybridizations from barley and to the barley transcriptomic atlas – a data set available for the research community that was generated from two barley genotypes, "Morex" and "Golden Promise," using a diverse series of tissues [53]. The WebComparator (http://contigcomp.acpfg.com.au/) is a web-based graphical interface that permits comparison of expression profiles of homologous genes across a wheat and barley tissue series. The search can be done for "probe name" or "function" and results include the homologous cluster relevant to the query probe set, a comparison of expression profiles and annotations [53, 122]. Genevestigator V3 (http://www.genevestigator.com) is another Web-based resource with diverse applications, from cancer and other human diseases to the model plant *Arabidopsis*, to crop plants. It offers a unique collection of gene expression data for a variety of commercial crop species (maize, wheat, rice, barley, and soybean), together with a set of specifically developed analysis tools that are accessible online [123].

The transcript profiling approaches based on hybridization are quite high throughput and relatively inexpensive. However, several limitations of these methods can be observed such as reliance upon existing knowledge about genome sequence, a fact for which they could be named "closed" systems; possible high background levels due to cross-hybridization; and a limited dynamic range of detection given by both background and saturation of signals. Moreover, comparing expression levels across different experiments can be difficult and requires sophisticated normalization methods [118, 119].

To overcome these drawbacks, in contrast to microarray methods, sequence-based approaches directly determine the cDNA sequence and for this reason they could be named "open" systems. Different techniques were developed and used in barley, including cDNA-AFLP [124], serial analysis of gene expression (SAGE) [125], massively parallel signature sequencing (MPSS), and "iGentfier," which combines elements of tag sequencing such as SAGE or MPSS and fragment display [126], allowing analysis of samples in high throughput using capillary electrophoresis equipment. Recently, the NGS methods led to development of a new approach for both mapping and quantifying transcriptomes called RNA-Seq (namely, RNA sequencing) that is expected to revolutionize in the short term the transcriptomic research. Total RNA is converted by reverse transcription (RT) into cDNAs with adapters attached to one or both ends. The output of RNA-Seq is usually composed of millions of reads of 30–400 bp, depending on the DNA sequencing technology used. Any high-throughput sequencing technology can be utilized for RNA-Seq, and the Illumina IG1, Applied Biosystems SOLiD, and Roche 454 Life Science systems have already been applied for this purpose, as reviewed by Wang *et al*. [118]. The reads resulting from the sequencing are either assembled *de novo* without a reference

genomic sequence or aligned to a reference genome, or to reference transcripts, to produce a genome-wide picture of transcription of both the level of expression and the transcriptional structure for each gene [118]. RNA-Seq does not necessarily depend on any prior sequence knowledge; there is no need for design of probes that must be based on prior sequence or secondary structure information. Therefore, this feature makes this method particularly attractive for nonmodel species. The technique has also other technical, expected advantages, such as higher sensitivity and greater range of detectable expression. Moreover, it has been demonstrated that 454-based transcriptome sequencing is a valid method for the high-throughput identification of gene-associated SNPs [127]. This technique has been applied to several plant species, from models *Arabidopsis* [128] and *Medicago truncatula* [129], to horticultural and field crops, for instance, cucumber [130] and soybean [131], either to study the gene expression and genome annotation or to create an organism-wide gene expression atlas. As regard the application of this technique to studies on stress tolerance, the use of 454/FLX sequencing of *Gossypium* transcriptome in genotypes characterized for contrasting drought tolerance by Asif and colleagues was reported by Thakur and Varshney [132], who reviewed challenges and strategies for NGS. Asif's analysis involved comparison of aligned tags using R statistics, followed by PCA and Biplot analysis. Several activities of the IBSC aim at extensive RNA-Seq in barley, as reported in the minutes of the IBSC Business Meeting at Plant Animal Genome XVIII, January 2010 [133]; however, still no published data are available.

The development and the use of efficient bioinformatic tools for analysis of transcript profiling data is an important issue that deserves a separate discussion, but not addressed here for space constraints. The availability of all these platforms, not only in *Arabidopsis* but also in crops like barley, led to significant studies of response to various abiotic stresses in the species, as reviewed here, although, as underlined before, mostly under single stress conditions.

34.2.4.2.2 **Transcriptional Profiling of Cold Stress in Barley** A set of EST sequences, obtained from a cDNA library of cold acclimatized leaves of cultivar "Nure," was delivered to the ITEC [51, 134]. The EST sequencing work, performed on a non-normalized cDNA library from cold-treated leaf tissue of a cold-tolerant variety, already showed, in a sort of *in silico* expression profiling experiment, that about 8–10% of the randomly chosen and sequenced cDNA clones from the leaf tissues had significant levels of homology with known *COR* (cold regulated) or other stress-related genes. A cDNA microarray developed from that collection of EST clones was thus used for expression profiling to more understand the cold acclimation genomic response [135]. Sequence alignment of the differentially expressed cDNA clones indicated that the most abundant *COR* mRNAs identified during EST sequencing were similar to *COR413*, *BLT4*, and *BLT14* gene families, and together represented about 4% of the nonnormalized barley transcriptome at low temperature [134]. These gene families were among the upregulated redundant clones identified in the microarray experiment. Different stress conditions (two cold treatments, three dehydration treatments, salinity, high light, and two copper treatments) imposed as single stresses, although including a combination of abiotic stresses, were studied

using a second cDNA microarray, consisting of more than 300 selected sequences directly or indirectly related to abiotic stress response [136]. Clones were selected on the basis of several previous publications concerning stress-related sequences, for instance, some ITEC-derived ESTs, or because they were involved in metabolic pathways known to be affected by abiotic stresses (sugar, lipid, and amino acid metabolisms) or because they belonged to families known to be involved in plant response to environmental factors, for instance, CBF-like and other AP2-related transcription factors. When the cold-tolerant cultivar "Nure" was challenged with a combination of stresses, the response was unique and different from that when two stresses were applied singularly, as it was demonstrated by Rizhsky *et al.* [13] in *Arabidopsis* by an oligo array experiment. In response to low temperature and in the presence of light, the repression of LHC (light harvesting complex) was observed coupled with the upregulation of genes coding for enzymes involved in the amino acid metabolism. On the other hand, genes coding for enzymes involved in the pathway leading to proline synthesis (P5CS – δ-1-pyrroline-5-carboxylate synthetase – and ornithine aminotransferase) were found upregulated after cold treatment independent of a light. The comparative analysis of the abiotic stress response in barley pointed out the existence of a group of three cold-specific sequences (dehydrins: *DHN5*, *DHN8*, and *COR14b*) induced only at low temperature and in all cold treatments, regardless of the presence of light or copper; these sequences could be considered as markers of the barley low-temperature response. Finally, the induction of different genes, such as some dehydrins, or proteins with heavy metal binding domains that are induced under many stress conditions, suggested a general role of a cluster of genes in abiotic stress response [136].

To identify barley genes involved in response to low temperature, to investigate the interactions among stress responses to cold and drought, and to specifically test the hypothesis that the dehydrin (*DHN*) multigene family can serve as an indicator of the entire transcriptome response, the responses of the cold-susceptible cultivar "Morex" to low temperature including chilling, freeze–thaw cycles, and deacclimation over 33 days, as well as to gradual drought was studied with Barley1 GeneChip [137]. The results obtained indicated that the majority (50%) of transcriptome changes at low temperatures are part of a general stress response common to chilling and freezing, while only smaller parts were specific either to chilling or to freezing. Since the intersection between frost- and drought-responsive genes was bigger than that of chilling- and drought-modulated genes, this suggested an important element of osmotic stress response to freezing stress condition. Three clusters of dehydrins with similar expression patterns were identified by hierarchical clustering analysis; those upregulated by drought stress but not by low temperature, those induced by both drought and low temperature, and the third cluster that did not respond to low temperature or drought, as already reported [136, 137]. Genes involved in biogenesis of cellular components, cell cycle, and DNA processing, protein modification, and destination and cellular transport were among genes induced exclusively by low temperature. Some of these changes were thought as probably related to mechanical stress induced by ice crystal formation in plant tissue. The mechanisms for cell rescue and defense including oxidative stress responses were activated. Genes

induced in response to both drought and low temperature included those involved in osmotic stress signal perception such as genes encoding phospholipid biosynthetic enzymes and calcium signaling proteins. Moreover, changes in transcripts involved in protection of membranes and proteins (accumulation of osmoprotectants such as galactinol and proline), genes coding for proteases and protease inhibitors important for protein homeostasis, and genes coding for polyamines were observed [137].

Transcription profiling of either genetic mutants or transgenic plants represents a powerful tool for dissecting the derived molecular networks. Barley genetic stocks offer a unique collection of chloroplast-deficient mutants, most of them characterized at genetic and biochemical levels. Thanks to them, the involvement of the chloroplast in the signal transduction pathway of the cold stress was demonstrated [138]. Those results were confirmed by wide-scale transcriptional profiling by the Barley1 GeneChip of cold-acclimated wild-type barley versus four chloroplast-defective mutants. Transcript profiling showed that 66.9% of the wild-type cold-regulated genes were either not or not correctly cold regulated in the mutants demonstrating that the chloroplast has a major role in the control of molecular adaptation to cold. Moreover, a fully operational chloroplast rather than a specific step in chloroplast development is required for normal cold-dependent regulation of the transcriptome. Three main pathways of the wild-type cold-regulated genes were identified: cold-regulated genes that were unaffected by any chloroplast mutation (including the *CBF*–C-repeat binding factor; transcription factors; and many genes known to be under *CBF* control), cold-regulated genes constitutively induced in all mutants (although to different levels), and finally, cold-regulated genes belonging to signaling pathways disrupted in all mutants, whose expression consequently was not (or only marginally) responsive to cold. These results brought the conclusion that the factors derived from the chloroplast in addition to CBFs are required to promote the full suite of molecular changes associated with cold acclimation [139] to withstand frost.

34.2.4.2.3 **Transcriptional Profiling of Drought Stress in Barley** The experimental conditions that have been applied in drought stress transcriptional profiling studies differ substantially as far as the dynamics and/or intensity of the water stress treatments applied are considered. Therefore, it is important to verify the correspondence of changes in expression profiles occurring under different experimental conditions mimicking drought conditions in the field [140]. In most cases, expression profiling for the response to water deficit has been performed on plants subjected to high-intensity stress treatments, frequently applied in a very short time, the so called "shock-like" (DHSH) treatments [136, 141]. Under such conditions, examples of significantly drought-affected genes in barley leaves, induced by stress, were those involved in the jasmonate biosynthesis (e.g., AOS – allene oxide synthase), together with the jasmonate-regulated genes (jasmonate-induced proteins – JIPs – and methyl-jasmonate-inducible lipoxygenase). Two arginine decarboxylases were induced (also in roots), possibly reflecting the altered synthesis of polyamines. LEA/dehydrin protein and metallothionein encoding transcripts, as well as P5CS involved in proline biosynthesis and some ABA (abscisic acid)-responsive proteins, were also found to be induced. In turn, most drastic downregulation was observed for

photosynthesis-related genes [141]. Dehydration was also shown to regulate several genes involved in glutathione biosynthesis pathway [136].

The use of "shock-like" treatments, however, may prevent the identification of some long-term responses that might play an important role in plant adaptation to drought under field conditions. A cDNA microarray mainly derived from dehydration-shocked barley leaf tissues was used to monitor expression changes in leaves of barley plants subjected to slow drying conditions in soil and after rehydration. The results were compared with those obtained under shock-like conditions (DHSH), showing changes in expression profiles according to the dynamics of the dehydration treatment [140]. The majority of the transcripts were, in fact, regulated only by one of the drought treatments, and the gradual dehydration of leaves led to a lower number of differentially regulated transcripts, with lower fold changes in expression levels when compared to the DHSH treatment. The changes in gene expression observed under DHSH were in agreement with results of other authors obtained under shock conditions [141]. Only a small portion of transcripts showed similar changes regardless of the shock-like/gradual type of water stress. These few common genes included upregulated genes coding for metallothionein-like proteins, some involved in jasmonic acid (JA) biosynthesis and in osmoprotectant synthesis. The results suggest that caution should be exerted when transcriptome information obtained under conditions of water deficit induced in a very short time is used to understand how plants may regulate gene expression in response to a water deficit developed under more naturally induced drought conditions. The same caution should be used when the same information would be aimed to identify candidate genes for QTL of field-related traits with an adaptive role in drought. Ideally, a correct strategy could be to carry out both types of treatments in time course experiments for a recording of transcript dynamic changes through the different conditions of water deficit [140].

Different experiments on barley response to drought stress have been conducted using the Barley1 GeneChip since the first study by Close [142], where the response of "Morex" barley to water deprivation stress was analyzed at different stages of stress. Barley1 GeneChip profiling of barley response to drought and cold enabled identification of genes responsive exclusively to drought among those involved in transcriptional regulation, including basic leucine zipper (bZIP) proteins, homeodomain-leucine zipper (HD-ZIP), and MYB family members [137]. The changes in the expression of transcription factors were mostly in the early stages of the treatment modulation of transcription [142]. Genes for downstream protection mechanisms induced by drought stress included those coding for several LEA proteins that are typically induced by osmotic stress or exogenous application of ABA. Dehydrin genes were reported to be expressed mainly during the most severe stages of the stress treatment [142]. Heat shock protein (HSP) genes, generally involved in cell rescue and defense mechanisms, were also induced. The induction of genes by combined drought and cold reported by Tommasini *et al.* [137] has been already reviewed above.

The study by Close [142] was performed, in the same manner as the one by Tommasini *et al.* [137], on the cultivar "Morex," cold susceptible, and not drought tolerant. In order to investigate the nature of a tolerance response to drought stress, expression profiling of the tolerant versus susceptible varieties was investigated, for

example, in case of cDNA-AFLP profiling of wild barley genotypes [143]. One of the differentially expressed transcript-derived fragments (TDFs) was selected as a promising candidate gene for water stress tolerance, sequenced, and characterized. The deduced amino acid sequence of the corresponding gene (called *HSDR4-H. spontaneum* dehydration-responsive) was homologous to rice Rho-GTPase-activating protein-like (Ras homologue-GTPase-activating protein-like) with a Sec14 p-like (phosphatidylinositol/phosphatidylcholine transfer protein-like) lipid binding domain. Analysis of *HSDR4* promoter region enabled identification of a new miniature inverted repeat transposable element (MITE), and different putative stress-related binding sites for transcription factors such as MYC, MYB, LTRE (low-temperature-responsive element), and GT-1, suggesting a role of the *HSDR4* gene in tolerance to drought stress. Moreover, the *HSDR4* gene was mapped to the long arm of chromosome 3H between markers EBmac541 and EBmag705, in a region that had been previously shown to affect osmotic adaptation in barley [143]. A recent comprehensive transcription profiling of differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage with Affymetrix Barley1 GeneChip [144] enabled the identification of three distinct groups of drought-responsive genes. The first group consisted of genes expressed exclusively in drought-tolerant genotypes under drought stress, encoding proteins that may be involved in drought tolerance, through (a) controlling stomatal closure via carbon metabolism (NADP malic enzyme and pyruvate dehydrogenase); (b) synthesizing the osmoprotectant glycine-betaine (CSMO – C-4 sterol methyl oxidase); (c) generating protectants against reactive oxygen species (aldehyde dehydrogenase and ascorbate-dependent oxidoreductase); and (d) stabilizing membranes and proteins (heat shock proteins and DHN3). The second group was composed of genes abundantly expressed in tolerant varieties (versus susceptible ones), both under drought and control conditions, and thus possibly belonging to a constitutive resistance mechanism. They were coding for proteins involved in signaling, such as calcium-dependent protein kinase (CDPK) and membrane steroid binding protein (MSBP), antisenescence, and detoxification (glutathione transferase) pathways. Finally, a third group of genes including those encoding P5CS, protein phosphatase 2C-like protein (PP2C), and several chaperones were differentially expressed in all genotypes, both tolerant and susceptible, under drought; thus, they more likely represented general drought-responsive genes in barley.

Most genome-wide transcriptional profiling studies on drought stress in barley and in other cereals have focused on the response of vegetative organs [137, 140, 144]. However, drought affects all stages of plant development and the reproductive stage is the most sensitive in temperate cereal crops. Understanding how drought affects reproductive development is crucial for improving yield in environments characterized by terminal drought. This notwithstanding, the response of the spike transcriptome to drought stress has been largely overlooked. The photosynthetic organs of the spike are the major suppliers of carbon to the developing seeds, and barley varieties, truly tolerant to drought, have a better chance of achieving normal seed size when available water is reduced during grain filling. The transcriptomes of

lemma, palea, awn, and seed of barley were thus compared using the Barley1 GeneChip. The results obtained suggested that among the spike organs, the lemma and the palea could be more actively tolerant to drought stress compared to the awn [145].

One of the most common responses to drought stress is accumulation of osmolytes. What is particularly interesting, the proline synthesis pathway driven by arginase and ornithine aminotransferase, and not the P5CS, seems to be preferred in barley spike during drought stress [145]. A similar situation was reported by Atienza *et al.* [136] under shock-like drought stress conditions.

34.2.4.2.4 **Transcriptional Profiling of Salt Stress in Barley** Large-scale approaches including differential display [146] and different microarray platforms [141, 147, 148] have been employed to identify genes responding to salinity stress in plants. Differential display analysis enabled identification of salt-inducible genes expressed strongly in roots and rarely or weakly in barley leaves. These genes included those coding for proteins involved in signal transduction (e.g., MAPK – mitogen-activated protein kinase; transcription factors; receptor protein kinase; and PP2A-protein phosphatase 2A), typical stress tolerance genes (encoding glutathione reductase, thioredoxin-like protein, and heat shock proteins), and genes coding for membrane transporters, members of the P450 family, enzymes involved in RNA metabolism or function, and enzymes of sugar or amino acid metabolism [146].

A salt stress has at least two components: an osmotic one and an ionic one. Therefore, studies should try to distinguish the effects of osmotic components on global gene expression from that of the ionic stress components. Two microarrays composed of spotted cDNAs that included drought- [141] and salt-responsive [147] clones have been used to investigate the expression of salt- and drought-induced genes in barley leaves and roots. In the former study, however, probably due to pronounced natural salt tolerance of the barley line used for experiments, and for the mild salinity stress conditions applied, a relatively small number of salt-regulated transcripts were identified; moreover, only a small number of transcripts showed overlap between drought and salinity response. Upregulated transcripts under both stresses included those enzymes of the biosynthetic pathway that lead to jasmonate (AOS), P5CS, and several transcripts known to accumulate under abiotic stress conditions such as metallothionein-like protein, early-responsive dehydration 1 (ERD1), or germin-like protein. Common downregulated transcripts included transport proteins such as lipid transfer protein (LTP) and an ATP binding cassette (ABC) transporter, while increase in ubiquitin-related transcripts in roots characterized the salt stress response [141]. The results of Ueda *et al.* [147] demonstrated that the majority of 52 genes differentially expressed under pure osmotic stress were differentially expressed also under salt stress. Some of those genes such as the upregulated genes for P5CS, betaine aldehyde dehydrogenase 2 (BDH2), and plasma membrane protein 3 and the downregulated genes for water channel 2, heat shock protein 70, and phospholipase C were involved in a wide range of metabolic and signaling pathways, suggesting that during the initial phase under salt stress, several cellular responses are mediated by changes in osmotic potential [147].

Barley1 GeneChip was used to investigate the transcriptional response of the barley cultivar "Morex" to a salinity stress applied at seedling stage [148]. The level of stress was similar to the conditions found in agricultural systems in California, and the stress was imposed gradually over a 4-day period. This enabled reduction of the osmotic effect of salt stress, which is most important during the early stages of stress imposition, and allowed to focus the study on the ion-specific effects of salinity on gene expression, as opposed to osmotic shock effects. Some genes known to respond to other abiotic stresses including dehydration, osmotic stress, and exposure to low temperature were modulated, such as *DHN5*, dehydration-responsive gene *RD22*, late-embryogenesis abundant *LEA*, low-temperature-induced protein blt101/salt stress-induced ES13, a cold-regulated protein 2, and a CCAAT-binding transcription factor (*CBF-B/NF-YA* – CCAAT-binding transcription factor subunit B/nuclear transcription factor Y subunit A). The largest group of induced genes related to abiotic stress falls into the category of heat shock proteins. Genes coding for P5CS and phosphoethanolamine *N*-methyltransferase, two rate-limiting enzymes for accumulation of proline and glycine-betaine, respectively, were induced at all time points indicating that osmoprotection appears to be one of the mechanisms triggered in barley as a relatively early response. In contrast to the first functional studies, fewer genes responding to salinity stress were identified, likely due to the moderate level of stress and its gradual application; moreover, only few genes common with previous studies [141, 147] were detected. The differences could be explained by the different platform used for analysis, by the different genotypes of barley used, and by the significantly different stress conditions applied, long-term salinity stress [147] versus salt shock [141], versus early stage of gradual and moderate stress application [148]. A particularly noteworthy feature of the early-salinity stress response of barley at seedling stage was the change in expression level of genes related to JA biosynthesis such as *AOS*, lipoxygenase (*LOX*), allene oxide cyclase (*AOC*), lipase (phospholipase) together with JA-responsive genes such as those coding for jasmonic acid-induced proteins, hordothionins, *O*-methyltransferase (*OMT*), glutathione *S*-transferase, and selenium binding protein [148]. The JA pretreated salt-stressed plants were reported to accumulate strikingly low levels of Na^+ in the shoot tissue compared to JA untreated salt-stressed plants, still after several days of exposure to stress, and a pretreatment with JA was shown to alleviate the photosynthetic inhibition caused by salinity stress [14]. Expression profiling after a short-term exposure to salinity stress indicated a considerable overlap between genes regulated by salinity stress and JA application. Three JA-regulated genes, arginine decarboxylase, ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) activase, and apoplastic invertase, were reported to be possibly involved in salinity tolerance mediated by JA [14].

In order to elucidate the mechanisms of salt tolerance in barley, the level of polymorphisms and differences in responses of two genotypes that differ in tolerance to salt stress were studied with Barley1 GeneChip [149]. "Golden Promise" accumulated lower shoot Na^+ compared to "Maythorpe" when growing under saline conditions and it was identified as salt tolerant. Such a response of salt tolerance in "Golden Promise" had been attributed to a single mutation at the *Ari-e* locus (on chromosome 5H) resulting from irradiation of "Maythorpe." However, the

expression profiling of salinity stress response of the two varieties was significantly different, with this difference being more apparent in the root comparison. Several of the JA biosynthetic pathway genes (*AOS*, *LOX2*, *LOX3*, and *OPR2* – oxophytodienoate reductase) were downregulated in response to salinity in "Maythorpe," while others like *AOC* and two JIPs were upregulated in "Golden Promise" but not in "Maythorpe" in response to stress, confirming previous results of JA involvement in salt stress resistance [14, 148]. JA appears thus to be an important component of heritable salt tolerance in "Golden Promise" and in barley in general. Two exchanger proteins were identified to be downregulated in "Maythorpe" roots in response to salinity stress (a Na^+/Ca^{2+} exchanger protein and a vacuolar cation/proton exchanger), and this might explain the low Na^+ accumulation trait of "Golden Promise," as salt tolerance in Triticeae is generally associated with Na^+ ion exclusion [150]. Only a low number of probes that are modulated in common in roots and shoots of two barley genotypes, corresponding to genes coding for P5CS, LTP, phosphoethanolamine cytidyltransferase, and dehydrin 7, probably take part in a general response of barley to salt [149].

A comparative functional genomics approach was successively applied to study barley response to salinity stress, to identify analogous and contrasting gene expression patterns between rice and barley, by using cDNA [151] and oligonucleotide microarray platforms [152]. Rice and barley are both members of Poaceae family, but they have a marked difference in salt tolerance. The differential regulation of a relatively larger number of genes in barley roots than in rice roots during the initial phase of salt stress suggested that barley root cells are more responsive to salt stress signals and that the divergent transcript profiles reflect fundamental differences in the biological responses to salt stress of those two species. The coordinated regulation of early synthesis of osmoprotectants proline and betaine, by *P5CS* and *BADH2* (betaine aldehyde dehydrogenase) genes, respectively, with regulation of water permeability (downregulation of water channels) could constitute a counterbalancing response to the initial decline in leaf water potential in barley under salt stress [151]. Divergences in rice and barley responses were confirmed by Barley1 GeneChip analysis of two rice and two barley susceptible and resistant genotypes [152]. Clustering analysis of differentially expressed genes revealed that there were no prominent gene clusters in rice and barley that had conserved expression patterns, while gene clusters with contrasting responses between barley and rice orthologues were identified. Genes involved in low-temperature response and acquired tolerance to freezing (*LOS2* – low expression of osmotically responsive gene 2), and in the H_2O_2-mediated programmed cell death (phospholipase Dδ), were among those upregulated in barley but repressed in rice. Activation of *PLDδ* decreases H_2O_2 cell death and improves stress tolerance. Genes related to cell wall biogenesis were repressed in barley, since high salt levels around roots could be expected to retard growth and cell wall biogenesis in the root tissue, while upregulated in rice. Moreover, several auxin-related genes were responsive, being downregulated in barley, but only to a small extent in rice. To increase the power of the comparative analysis approach, a data set generated in wheat roots during salt stress using the Affymetrix Wheat Array was chosen for *in silico* comparison. The overall

results showed that the transcriptional response to high salt is conserved to a significant degree between wheat and barley. But it is highly divergent in rice [149].

34.2.4.2.5 **Transcriptional Profiling of Nutrient and Metal Stress in Barley** Nitrogen is a crucial macronutrient required by plants, and development of crop varieties with high nitrogen utilization efficiency (NUE) adapted to low input management systems is mandatory for sustainable agriculture. The content of nitrogen intermediates is highly influenced by nitrogen availability. Moreover, nitrogen-containing compounds play an active role as metabolic scavengers of excess ammonium accumulated under stress [153]. Understanding the plant response to different nitrogen conditions is essential for the development of future approaches for NUE improvement. Notwithstanding, nitrogen deficiency stress is the most important nutrient stress limiting barley grain yield worldwide; a survey of bibliography did not recover, to our knowledge, any significant result in genome-wide functional studies in this species. Microarray and RNA gel blot analyses were performed to identify *Arabidopsis* genes responding to both low and high nitrate concentrations [154]. Genes associated directly or indirectly with nitrite reduction were most highly induced. Most of the known nitrate-regulated genes, including those encoding nitrate reductase, the nitrate transporter (NRT1), and glutamate synthase, were induced too; moreover, some other nitrate-induced genes were also found, including those encoding regulatory proteins, metabolic enzymes, such as transaldolase and transketolase of the nonoxidative pentose pathway, and proteins with unknown functions. Two genes, *AMT1* encoding an ammonium transporter and *ANR1* encoding a MADS-box factor, were repressed by nitrate. A high-density array of mineral nutrition-related cDNAs was used to study the response of tomato plants exposed to nitrate deprivation at different time points after nitrogen was withheld for 48 h [155]. One hundred and fifteen genes were found to be upregulated by nitrate resupply. Among these genes were included several previously identified as nitrate responsive, including nitrate transporters, nitrate and nitrite reductase, and metabolic enzymes such as transaldolase, transketolase, malate dehydrogenase, asparagine synthetase, and histidine decarboxylase. Moreover, some other nitrate-inducible genes, such as those coding for water channels, phosphate and K^+ transporters, genes potentially involved in transcriptional regulation, some stress response genes, and ribosomal protein genes were found induced. In wheat, the activity of three different enzymes involved in nitrogen assimilation, GS (glutamine synthetase), GOGAT (glutamine synthetase oxo glutarate), and GDH (glutamate dehydrogenase) under different nitrogen treatments, was analyzed and compared with gene expression profile of a Dof (DNA binding with One Finger) transcription factor. The elevated expression of *Dof 1* at the grain filling stage was proposed to be responsible for overexpression of the GS and GOGAT genes, thereby prolonging their activities [156]. As far as barley is concerned, the studies reported the isolation of the first *HvNRT2.1* and *HvNRT2.2* genes encoding putative NO_3^--inducible high-affinity transporters in higher plants [157]. Northern blot analysis of barley plants exposed to nitrogen starvation showed that the *HvNRT2* transcript accumulated rapidly in roots after NO_3^- was supplied [157]. Vidmar *et al.* [158] reported the isolation of two new cDNAs, *HvNRT2.3* and

HvNRT2.4, which are closely related to *HvNRT2.1* and *HvNRT2.2*. Moreover, the correlation between Northern blot patterns of the *HvNRT2* gene expression and $^{13}NO_3^-$ influx in response to various nitrogen sources confirmed the identification of the *HvNRT2* genes as participants in high-affinity NO_3^-. In a study by Wang *et al*. [159], a correlation of the level of 6-SFT (sucrose:fructan 6-fructosyltransferase) mRNA determined by Northern blot analysis with fructan accumulation induced by nitrogen deficiency in barley leaves was demonstrated suggesting that the changes in fructan levels under N stress are connected to the fructan synthesis rate that is controlled by 6-SFT.

Transcriptomic analyses in *Arabidopsis* [160], rice [161], and lupin [162] have shown that coordinated changes in the expression of numerous genes involved in various metabolic pathways, such as photosynthesis, carbon metabolism, nitrogen assimilation, and synthesis of protein and nucleic acids, take place in P-deficient plants. As regard barley no genome-wide transcriptional studies for functional genomics were reported.

Aluminum-resistant cultivars of barley rapidly secrete citrate from the roots in response to Al, and a good correlation between Al resistance and the amount of citrate secretion among different cultivars was reported. Microarray analysis with Barley1 GeneChip enabled identification of a candidate gene, *HvAACT1* (aluminum-activated citrate transporter) belonging to the multidrug and toxic compound extrusion (MATE) family, whose expression is constitutively higher in tolerant cultivar than in the susceptible one. The *HvAACT1* expression was shown not to be induced by Al exposure in neither of cultivars suggesting that it is constitutively expressed in the roots and that the secretion of citrate is mediated through the activation of HvAACT1 [163]. Four SNPs were found in the ORF of *HvAACT1* in 10 barley cultivars differing in Al resistance, but these SNPs could not explain the differential citrate secretion. In contrast, a good correlation was found between the expression of *HvAACT1* and the amount of citrate secretion in these cultivars. These results indicate that higher expression of *HvAACT1* rather than its sequence polymorphism is required for greater release of citrate. A recent study in wheat demonstrated that the expression of *ALMT1* (Al-activated malate transporter 1) may be controlled by the presence of the sequence repeats upstream of this gene. Furukawa *et al*. [163] suggest that the expression of *HvAACT1* might also be controlled by the promoter regions, a hypothesis that has to be investigated.

Microarray analysis with Barley1 GeneChip proved that barley plants survive in the presence of the toxic heavy metal $HgCl_2$ (mercuric chloride) through several mechanisms that include water uptake limitations, shoot and root growth regulation, increased expression of genes involved in the biosynthesis of several plant protection secondary metabolites, and finally through detoxification. Genes involved in detoxification and defense mechanisms induced in the presence of mercury include several cytochrome P450s, glucosyltransferases and glutathione *S*-transferases together with genes involved in amino acid metabolism (experiment submitted by Lopes to PLEXdb, 2009 [164]).

Genes coding for protein with heavy metal binding domain (metallothionein-like protein and heavy metal binding farnesylated protein), whose induction is common

to different stress responses have been found induced in response to the plant micronutrient copper (Cu) [136]. Excess copper stress conditions promoted a strong upregulation of genes coding for antioxidant enzymes such as peroxidase and ascorbate peroxidase, underlining the presence of a high oxidative stress. In fact, copper, although an essential metal, is also capable of catalyzing the formation of reactive oxygen species that can cause intracellular oxidative damage to lipids, proteins, and nucleic acids [136].

Both limiting and excess (toxic) soil concentrations of the essential micronutrient boron (B) represent major limitations to crop production worldwide. *Bot1*, a *BOR1* efflux transporter orthologue, was identified as the gene responsible for the superior boron toxicity tolerance of the Algerian barley landrace "Sahara" and located at the *Bot1* tolerance locus by high-resolution mapping. Compared to intolerant genotypes, "Sahara" contains about four times as many *Bot1* gene copies, produces substantially more *Bot1* transcripts, and encodes a BOT1 protein with a higher capacity to provide tolerance in yeast. *Bot1* transcript levels identified by QRT-PCR in barley tissues are consistent with a role in limiting the net entry of boron into the root and in the disposal of boron from leaves via hydathode guttation [165]. Barley1 GeneChip was then used to investigate transcriptome changes associated with boron toxicity in the susceptible barley cultivar "Hamidiye," revealing that B toxicity results in global changes in the barley transcriptome and involves networks of signaling or molecular responses [166]. Boric acid treatment resulted in modulation of genes involved in JA biosynthesis (most of them were upregulated) and JA-responsive genes (both upregulation and downregulation were observed). JA is one of the signaling molecules produced in an integrated signaling network and B toxicity might be inducing a response that is connected to the JA regulated response. The induction of JA-related or -responsive genes as a key feature of response to salinity in barley was reported [148]. Ozturk *et al.* [141] reported upregulation of genes encoding JA-responsive proteins under drought stress in barley. Moreover, boric acid-induced expression of genes encoding glutathione *S*-transferase, pathogenesis-related or senescence-associated proteins. Expression of genes coding for transcription factors, chaperones, and transport proteins were modulated [166].

A microarray containing 8987 rice ESTs was used to analyze the gene expression profile of barley roots grown under either low zinc (Zn) or low iron (Fe) stress. Inductions in expression of genes involved in the methionine cycle in both Zn-deficient and Fe-deficient barley roots were reported [167]. It was reported that Zn deficiency increases the secretion of metal chelators (MAs) that are known to be produced in grasses in response to iron deficiency. The levels of the *HvNAS1* (nicotianamine synthase), *HvNAAT-A*, *HvNAAT-B* (nicotianamine aminotransferase), *HvIDS2*, and *HvIDS3* (iron deficiency-specific) transcripts, which encode the enzymes involved in the synthesis of MAs, were increased in Zn-deficient roots. Studies on the genes involved in the methionine cycle showed that the transcripts of these genes were increased in both Zn-deficient and Fe-deficient barley roots, probably allowing the plant to meet its demand for methionine, a precursor in the synthesis of MAs. Analysis using the positron-emitting tracer imaging system (PETIS) confirmed that more ^{62}Zn(II)-MAs than $^{62}Zn^{2+}$ were absorbed by

the roots of Zn-deficient barley plants. These data suggest that the increased biosynthesis and secretion of MAs arise from a shortage of Zn and that secreted MAs are effective in absorbing Zn from the soil [167].

The most important result of expression profiling studies, from an application point of view, is the identification of candidate genes with an effect on tolerance or resistance. Such candidate genes, when coincident with quantitative trait loci (QTL) that have a significant effect in increasing stress tolerance under field conditions, are reinforced in their role as actors for real abiotic stress tolerance. In turn, this information may offer additional opportunities for a more effective application of marker-assisted selection (MAS), genetic engineering, and/or other genomics approaches for the release of stress-resistant cultivars.

34.2.5
Quantitative Genetics and Genetical Genomics of Abiotic Stress Tolerance

Plant tolerance to abiotic stress is largely grounded on quantitative genetic bases [168]. For this reason, the discovery of quantitative trait loci, controlling the genetic variation in the tolerance traits, and their manipulation via MAS are very important for obtaining the successful integration of genomics approaches in breeding programs. Since the first proof-of-concept of Sax in 1923, which demonstrated in bean the possibility to identify a genetic locus associated with the variation in a quantitative trait, plant science had to wait many decades for the availability of appropriate algorithms in the late 1980s, and of genetic molecular maps in the early 1990s. These two developments allowed plant scientists to start mapping the genomic regions that have a statistically significant impact on the quantitative variation of agronomically relevant traits. QTL mapping, from a conceptual point of view, represented an enormous breakthrough for plant biology since it made possible the dissection of the complex inheritance of quantitative traits, until that time only under speculative research, into "Mendelian-like" factors. In such a state, they could be identified, numbered, characterized, and validated, and were amenable to be selected through the linked flanking molecular markers and, eventually, to be cloned. This last significant step forward was achieved for cultivated plants in the first decade of this century.

Many studies in barley as in other cereals described important QTL imparting tolerance to adverse environmental stressors. These, as other many QTL responsible for pathogen quantitative resistance, quality traits, and yield, could be named today "phenotypic" QTL (phQTL). In fact, the two key elements to get an accurate genomic placement of quantitative phenotypic trait-driving loci are the availability of a complete and robust genetic map, together with a careful phenotyping for the trait (validated in more than a single experiment) of the experimental population that gave origin to the genetic map. The classical phQTL mapping approach is then a linkage mapping approach, in which the count of recombinants between marker loci takes to the genetic map construction, and the so-called "interval mapping" procedure [169] scans the genome by intervals, assigning a probability for the allelic variation at each interval to be associated with a trait variation. The statistical methods to calculate such

a probability score or, better, logarithm-of-the-odds (LOD) score evolved from the initial ML (maximum likelihood) mapping algorithm [169] to the regression mapping algorithm [170, 171]. QTL linkage mapping was joined in the past decade by QTL association mapping, named LD (linkage disequilibrium) mapping. With such a procedure, an LD-based genetic map, often starting from an integrated genetic map coming from different linkage mapping projects, is used to associate the allelic variation at the mapped loci with the phenotypic variation, both scored on an unrelated plant population. The first publication on the usage of LD in barley to explore the genome for mean yield and yield adaptability QTL is from Kraakman *et al.* [172]. The authors simply used Pearson correlation coefficients to evaluate the marker-trait associations and to derive the positions of QTL on an integrated barley genetic map.

To date, more than 1200 studies on plants have been published on mapping phQTL [173]. Such a wealth of information has been stored for barley in web-based resources such as mainly Graingenes (http://wheat.pw.usda.gov/GG2/index.shtml [174] and, although with few data, Gramene (http://www.gramene.org [175]), and Cerealab (http://www.cerealab.unimore.it [176]). These databases, however, also contain other genomics information, such as genetic maps, molecular markers, and DNA sequences. Interestingly, from Graingenes, directly accessible through the "Barley Boulevard," a "Barley QTL Community Curation Book" is downloadable as an MS Excel spreadsheet.

By merging and integrating diverse types of data and information across species and levels of biological complexity, the QTL databases enhance the potential to understand and utilize QTL information. Moreover, the accumulation of an impressive amount of phQTL data brought about the development of the concept of meta-analysis. "Meta-analysis" method enables to combine QTL results from different independent analyses, and was first described in general terms by Glass in 1976 [177]. This technique has been used mainly by researchers in medical, social, and behavioral sciences, and also in genetics and plant evolution (see, for example, Ref. [178]). Meta-analysis is an important tool in linkage analysis. The pooling of results across primary linkage studies allows greater statistical power to detect QTL and more precise estimation of their genetic effects and, hence, yields conclusions that are more conclusive than those of individual studies. The meta-analysis has, for example, been applied in rice to dissect the complex genetic structure of drought-related QTL, and in some cases was able to clarify the crucial question if overlapping loci displayed pleiotropy or close linkage between adjacent genes [179]. The main practical value of meta-QTL analysis is in the ability to provide an accurate estimate of QTL position that will be useful in marker-assisted selection and candidate gene identification [179]. The first attempt of QTL summary for abiotic stress tolerance in the Triticeae was made by Cattivelli *et al.* [180]; in this chapter, we propose an updated version of such a QTL survey, reported on a barley Bin-map [181] (Figure 34.1). The figure shows the "hot spots" of phQTL determining tolerance/adaptation to various abiotic stresses in barley, together with the approximate map positions of cloned genes underlying QTL, and likely candidates to explain tolerance components. It should be emphasized here the colocation between multiple QTL of abiotic stress

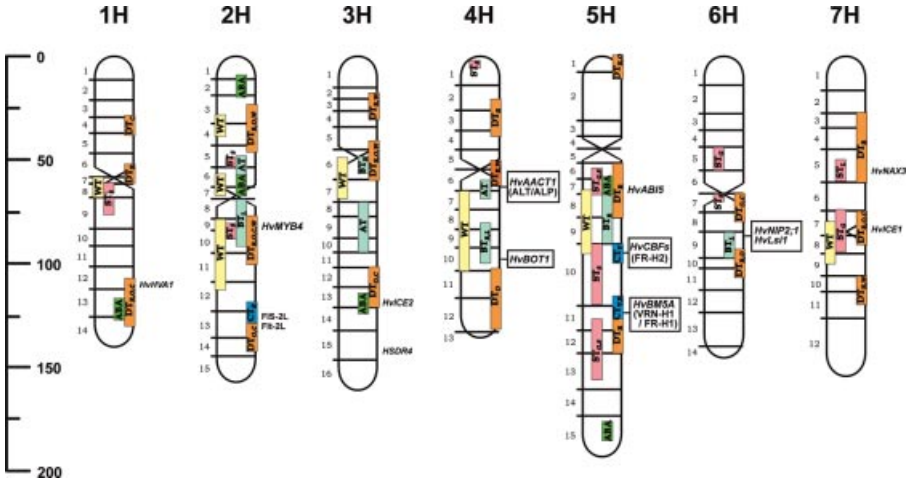

Figure 34.1 Summary of abiotic stress tolerance QTL mapped on the barley genome (see details in the text). Chromosome length, QTL map comparisons, and loci assignment to marker Bins are based on both the barley Bin map ([181] and http://barleygenomics.wsu.edu) and the consensus map of Marcel *et al*. [45]. Chromosome length is reported on the left in Kosambi cM. QTL: CT, cold tolerance at vegetative (V) and/or reproductive (R) stage; DT, drought tolerance measured as relative water concentration (R), osmotic potential/osmotic adjustment (O), carbon isotope discrimination (C), and water-soluble carbohydrate concentration (W); ST, salt tolerance at germination (G) or seedling (S) stage; BT, boron tolerance measured as root (R) and leaf (L) sensitivity; AT, aluminum tolerance measured as root elongation sensitivity; WT, waterlogging tolerance; ABA, abscisic acid response at germination. Cloned QTL, candidate genes to explain QTL, and major loci have been reported on the right of chromosomes. Their positions were derived from Graingenes (http://wheat.pw.usda.gov/GG2/index.shtml) and from the literature cited in the text. Gene names are reported in italics with the *Hv* prefix; major loci names in normal style; cloned QTL are in boxes.

tolerance and the two regulatory candidate genes *HvMYB4* and *HvABI5* on, respectively, Bin 9 of chromosome 2HL and Bin 7 of chromosome 5HL. *HvMYB4* maps in a region where loci for drought-related traits, waterlogging, boron toxicity, and salinity tolerance were discovered (see Section 34.2.5.2). Interestingly, the rice orthologous gene *OsMYB4* encodes an MYB transcription factor induced during exposure to low temperatures and other abiotic stresses [182]. *HvABI5* is a bZIP transcription factor, upregulated by ABA, which binds to *cis*-elements in the promoter region of the ABA- and stress-responsive COR/LEA effector genes *HVA1* and *HVA22* [183]. Notably, the involvement of *HvABI5* in abiotic stress tolerance was reinforced by the report of Kobayashi *et al*. [184], in which the authors found a positive role of a wheat *HvABI5* orthologue (namely, *Wabi5*) in response to low temperature, drought, and exogenous ABA treatment.

The accumulation in 20 years of a very large number of phQTL results for a wide range of traits and in the most important crop species led in the late 1990s to emerging concerns and discussions about some aspects of QTL mapping. Although

most of the worries have been solved (see, for example, Ref. [185]), a brief discussion about dependence on background effects, QTL × QTL, and QTL × E interactions deserves to be treated here since they are aspects of the genetic basis of quantitative traits that scientists and breeders have always to cope with. It is, in fact, quite obvious that different steps of a metabolic pathway leading to a phenotypic trait can negatively or positively interact; as it is quite obvious that allelic variants of a gene underlying a QTL, and present in a particular genotype, could act better, or exclusively, in a particular environment. Some studies dealt with the evaluation of both the effects, QTL × QTL and QTL × E, in barley [186], as in other plants. For example, Xu and Jia [187] evaluated the "Harrington" × "TR306" barley doubled haploid (DH) population "TR306" for QTL mapping in different environments for seven yield-related quantitative traits. They found that epistatic variance contributed to the genetic variance, but the cumulative contribution from significant epistatic effects was very small compared to that from the additive effects. The interaction between the genomic regions and the environment (QTL × E) has been investigated, for example, in a study by Comadran *et al.* [188] where the analysis of phenotypic and genotypic data for a different set of barley germplasm is described, with the phenotypic data being collected from yield trials grown across a wide range of Mediterranean environments subjected to varying levels of stress.

The existence of QTL interactions have both practical (MAS) and theoretical (genomics) implications; in the first case, they suggest to proceed for QTL identification in parallel with the breeding process. In the second case, they suggest the need to deepen the study of both simple and complex QTL networks, on a genome-wide basis.

34.2.5.1 Genetical Genomics of Abiotic Stress Tolerance

Since a QTL is by definition a genomic locus explaining part of the variation for any quantitative trait recorded in a plant population, the trait might be not only a field-recorded one but also the quantity of a protein, of a metabolite, or of a transcript. For example, in barley, a QTL for the accumulation of a cold-regulated (COR) protein, detected by Western blotting, was coincident with a QTL of frost tolerance [189]. If we extend this concept to a "omics" scale, we could outline the so-called strategy of "genetical genomics" [190]. Owing to such an approach, genetical genomics can generate in principle QTL maps from any method of genome-wide expression profiling based on mRNAs (eQTL), proteins (pQTL), or metabolites (mQTL).

An emerging application of transcriptome analysis is the identification of the so-called eQTL, namely, QTL that control the level of expression (hence the "e") of particular genes. Differentially expressed genes involved in metabolic or regulatory pathways and identified by means of high-throughput methods are treated as phenotypes, while allelic variants that underlie the gene expression changes are identified in genetically related lines. eQTL can thus be classified as *cis*- or *trans*-acting based on the location of the transcript compared to that of the eQTL influencing the expression of that transcript. eQTL that map to the same genetic location as the gene whose transcript is being measured generally indicate the presence of a *cis*-acting regulatory polymorphism in the gene (*cis*-eQTL). eQTL that map distant to the

location of the gene being assayed most likely identify the location of *trans*-acting regulators (*trans*-eQTL) that may control the expression of a number of genes elsewhere in the genome [191].

To date, the eQTL strategy has been successfully applied in sequenced model plants, such as *Arabidopsis* [192] and rice [193], and also in barley in response to *Puccinia hordei* infection [194]. However, genomic surveys of eQTL for abiotic stress tolerance are still rare, or at their very beginning, in crops [195, 196]. Although not related to abiotic stresses, an interesting eQTL discovery in barley has been reported by Potokina *et al*. [50]. The authors used the Affymetrix barley GeneChip to measure transcript abundance in embryo-derived tissue of germinating grains collected from 139 DH lines of the "Steptoe" × "Morex" population. A genome-wide QTL analysis based on expression data of about 16 000 transcripts was thus performed and more than 23 000 significant eQTL, affecting the expression of about 13 000 genes, were found [50]. Interestingly, several regions that had many more eQTL than expected by chance alone if one assumes a uniform distribution based on genes per centiMorgan were identified, and an eQTL hot spot on chromosome 5H Bin 1, affecting expression of more than 200 genes, were coincident with a major locus of grain hardness already mapped in the same population.

Expression QTL researches alone, however, may fail to dissect physiological traits. For instance, if a sequence polymorphism does not affect gene expression itself, although it leads to differences in protein stability, enzymatic activity, or metabolite production, an eQTL would not be detected at that locus. In these cases, as in general, the other genetical genomics approaches could be used for the parallel measurement of the abundance of thousands of proteins and metabolites, to map protein QTL (pQTL) and metabolite QTL (mQTL). Moreover, as another future development of the genetical genomics, transcript, protein, and metabolite profiling could be combined to extract the highest possible information from each data set. Unluckily, as far as we know, no or very few studies on protein or metabolic QTL mapping for abiotic stress tolerance/adaptation have been published in barley so far. A recent interesting example of the application of pQTL analysis in barley is the work of Witzel *et al*. [197]. The authors employed a two-dimensional gel electrophoresis to characterize the grain proteome deriving from a set of doubled haploid introgression lines (IL) in which the *H. vulgare* spp. *spontaneum* Hs213 represented the donor parent and *H. vulgare* spp. *vulgare* cv. "Brenda" the recurrent one. A total of 51 quantitative trait loci for protein expression (pQTL) were found. With the only exception of chromosome 3H, where no pQTL were detected, an evenly distribution of loci was observed. Mass spectrometry (MS) was also used to examine proteins underlying pQTL and the most expressed were those involved in metabolism and disease/defense-related processes [197].

In the past 2 years, several studies have been carried out at the metabolomic level in *Arabidopsis* and other most studied plant species, which have led to a far richer description of the natural variation of chemical composition in these species facilitating the identification of important sources of allelic variance for metabolic engineering. Meyer *et al*. [198] used gas chromatography–mass spectrometry (GC-MS) to study the primary metabolism of Columbia × C24 RIL population.

Although no single primary metabolite displayed a strong correlation with plant biomass accumulation, a metabolic signature composed of contributions from various metabolites was identified and abundance data allowed the detection of 157 metabolic QTL [198]. More recently, Rowe *et al.* [199] conducted a similar study on 210 RIL genotypes derived from the "Bayreuth" (Bay) × "Shahdara" (Sha) cross. The distribution of metabolite QTL across the genome included 11 QTL clusters; 8 of these clusters were associated with an epistatic network that regulated the plant's central metabolism. Since the same population was previously employed for global eQTL analysis, the authors compared the heritability of the mQTL they found with the results of West *et al.* [200] and showed that mQTL tended to be less heritable than the eQTL displaying a moderate phenotypic effect [199].

As a general consideration about the advancement of "omics" sciences in the study of quantitative variation, as well as of QTL mapping in general, such advancements could enable the cloning of most mapped QTL in plants. This will definitely finalize their mendelization, and move plant science to a step forward similar to that of the advent of QTL mapping. Another opportunity for the near future could be a better understanding of the QTL × QTL interactions on a genomic scale, together with the identification of the biological meaning for the QTL × E effects.

34.2.5.2 Genomic Regions Carrying phQTL for Abiotic Stress Tolerance

34.2.5.2.1 phQTL for Cold (Frost) Stress Tolerance As underlined in Section 34.1, Triticeae need to experience an acclimation period (hardening) to acquire a complete low-temperature resistance. In fact, it is during the hardening phase that the metabolic machinery of response to frost is activated [134, 201]. For this reason, functional genomics studies aiming to explore the key components of such molecular mechanisms have been conducted at low, nonfreezing temperatures (see, for example, Ref. [202]). On the other hand, as phenotypic evaluation for frost tolerance has a more applied significance, most QTL searches were done for tolerance to frost (Fr) conditions, both in the field and in the controlled environments. Although considered a polygenic trait, since the early 1990s, QTL mapping has led to the identification in barley of a relatively small number of quantitative trait loci having major effects on the ability of the plant to survive freezing (i.e., exposure to −11/−13 °C). Since the first reports, a large genomic region of chromosome 5H was highlighted as responsible for winter survival in the "Dicktoo" × "Morex" population, with one QTL mapped to the *VRN-H1* vernalization requirement locus [203, 204]. Later on, Francia *et al.* [189] developed the "Nure" (winter) × "Tremois" (spring) N × T linkage map and DH population as valuable genetic resources for the analysis of winter hardiness-related traits. They demonstrated that frost tolerance was controlled mainly by two linked QTL (*Fr-H1* and *Fr-H2*) rather than by one, both located on 5H, explaining a large part of phenotypic variation [189]. As far as we know, the N × T genetic system is the only population where both *Fr* QTL are segregating in the Triticeae since in wheat *Fr-A1* [205] and *Fr-A2* [206] were mapped in different genetic systems. It has also been shown by colinearity approaches that the two loci of chromosome group 5 are conserved in the Triticeae [207]. Tolerance at *FR-H2* appears

to be governed by a cluster of at least 12 *CBF* genes, which reside at this locus [54, 189, 208, 209], whereas *FR-H1* was found as overlapping with the *VRN-H1* vernalization response locus. At the moment, testing the hypothesis whether the effect of *Fr-H2* is either due to a "CBF number game", that is, due to a particular and critical number of *HvCBF*s acting in a coordinated manner, or due to the action of only one *HvCBF* is still underway. Knox *et al.* [210] showed that barley genotypes "Dicktoo" and "Nure," carrying a *vrn-H1* winter allele at *VRN-H1* harbor, increased copy numbers of *CBF* coding sequences in comparison to genotypes "Morex" and "Tremois" carrying a *Vrn-H1* spring allele. Sequencing bacteriophage lambda genomic clones from these four cultivars, alongside DNA blot hybridizations, indicated that approximately half the *CBF* orthologues at *FR-H2* are duplicated in individual genomes. One of these duplications discriminates winter (*vrn-H1*) genotypes from spring (*Vrn-H1*) genotypes, as the winter cultivars harbored tandem segmental duplications through the *CBF2A–CBF4B* genomic region and maintained two distinct *CBF2* paralogues, while the spring varieties harbored single copies of *CBF2* and *CBF4* [210]. An additional *CBF* gene, *CBF13*, was a pseudo-gene interrupted by multiple nonsense codons in "Tremois," whereas *CBF13* showed a complete uninterrupted coding sequence in "Dicktoo" and "Nure." Finally, DNA blot hybridization with wheat DNAs revealed that a greater copy number of *CBF14* also occurs in winter wheats than in spring wheats. Overall, these data indicate that variation in *CBF* gene copy numbers is widespread in the Triticeae and suggest selection for winter hardiness coselects winter alleles at both *VRN-1* and *FR-2*. To further reinforce the hypothesis on the main role played by the CBFs in the cultivated germplasm, in their work Tondelli *et al.* [54] mapped to chromosome 7H, loosely linked, two barley orthologues of the *AtFRY1* and *AtICE1* genes, putative upstream regulators of *CBF* genes in *Arabidopsis* [211, 212]. No QTL of cold tolerance were mapped on chromosome 7H, suggesting that allelic variation at these two CBF regulators are not important for the trait.

Vrn-H1 is one of the three vernalization genes loci required to determine barley growth habit [213, 214]. Limin and Fowler [215] suggested that the main vernalization gene in wheat, *VRN-A1 (VRN-1)*, which is induced during vernalization, plays an important role in decreasing the ability to cold acclimate with development. Since *FR-A1 (FR-1)* cosegregates with *VRN-1* in most genetic studies, until recently it was not clear if *FR-1* was an independent gene or just a pleiotropic effect of *VRN-1* [189, 216]. Notably, barley genotypes carrying the *vrn-H1* allele for winter growth habit express certain *CBF* genes at higher levels than genotypes carrying the *Vrn-H1* allele for spring growth habit [217]. Moreover, once the winter genotypes carrying the *vrn-H1* allele are vernalized, *CBF* transcript levels are dampened relative to levels detected in nonvernalized plants [217]. This suggests that *VRN-H1* somehow acts to repress expression of the *CBF*s at *FR-H2* and in turn decrease freezing tolerance. Recently, Dhillon *et al.* [218] used two diploid wheat (*T. monococcum*) mutants, maintained vegetative phase (*mvp*), which carry deletions encompassing *VRN-1*. The *Mvp/2* normal flowering plants showed reduced freezing tolerance and reduced transcript levels of several *CBF* and *COR* genes compared to the *mvp/mvp* never flowering plants. Diploid wheat accessions with mutations in the *VRN-1* promoter, resulting in

high transcript levels, showed a significant downregulation of *COR14b* under long days, but not under short days. These results suggested that *VRN-1* is required for the initiation of the regulatory cascade that downregulates the cold acclimation pathway. In the same study, Dhillon *et al.* [218] hypothesized that previously mapped *FR-1* on the chromosome 5A region was likely a pleiotropic effect of *VRN-1* rather than the effect of a separate closely linked locus.

Generally, in winter barley growing areas, frost is experienced at the vegetative stage of development. In Australia, the predominant frost damage occurs from radiation frost events in spring during the reproductive stages of barley and other cereals' development. Reproductive tissue is very sensitive and can be damaged by small subzero temperature drops ($\leq -2\,^{\circ}C$). QTL for reproductive frost tolerance were then identified by Reinheimer *et al.* [8] and by Chen *et al.* [219]. Reinheimer *et al.* [8] indicated that two chromosomes, 2H and 5H, were implicated in the genetic control of reproductive frost tolerance. The QTL on chromosome 2HL was identified for frost-induced floret sterility in two different populations at the same genomic location, named *Flt-2L*, which was not linked to previously reported developmental or stress-responsive loci. QTL on chromosome 5HL were identified for frost-induced floret sterility and frost-induced grain damage in all the three populations studied, coincident with *Fr-H1*/*Vrn-H1*. Similar results were obtained by Chen *et al.* [219]. Winter alleles at the *VR-H1* vernalization response locus on 5H were linked in coupling to reproductive frost tolerance.

34.2.5.2.2 **phQTL for Drought Stress Tolerance** Among the different abiotic stresses, drought is by far the most complex and devastating for crops on a global scale [220]. Because of its complex genetics, it is often considered the most difficult trait to improve. Drought stress might be defined as the condition in which the amount of water available for the plant or through rainfall or irrigation is not sufficient to cover the plant evapotranspiration demand [221]. As a consequence, the level of drought tolerance of a plant has been determined by a great number of traits over the past decades and also in barley, but not always physiologically meaningful. Jones [222], for instance, underlined how in the majority of the molecular studies, measurements of plant water status (e.g., relative water content, RWC) are often not made, although they would be needed for good and appropriate measurement of tolerance. However, final grain yield of a given genotype in drought environments, with equal length of growth cycle phases with respect to other genotypes, should be considered as the best target trait of tolerance (measured as adaptation to drought-prone areas) [188, 223]. In this view, in drought tolerance studies the time period of drought stress is also very important. The longer this period, the more drastic are the consequences on the yield. Moreover, the different crop developmental stages show different sensitivity to drought stress [224]. Adaptation of barley growth cycle, and corresponding variation at cloned genes/QTL driving such adaptation, is also extremely important for stable yields in drought-prone environments. Very recently, Francia *et al.* [225] showed that plant adaptation in terms of grain yield was primarily driven by the allelic constitution at three out of the four major candidate genes for flowering time segregating in the studied segregating

population (i.e., *Vrn-H1*, *Ppd-H2*, and *Eam6*). The interrelationships among a series of characters defining grain yield and its components were explored as a function of the length of the vegetative, reproductive, and grain filling developmental phases. In most of the 18 environments across the Mediterranean, the best performing (adapted) genotypes were those with faster development until early occurrence of anthesis; the study confirmed the crucial role of the period defining the number of grains per unit area in grain yield determination in Mediterranean environments.

In 2002, Ozturk *et al.* [141] identified a number of differentially expressed sequence tags (dESTs) and putative candidate genes for drought response. These genes and ESTs were later used by Diab *et al.* [226] to enrich the linkage map developed by Teulat *et al.* [227], in the "Tadmor" × "Er/Apm" barley cross. On this map, 68 phQTL for different phenotypic measurements of drought stress were identified [226], and the highest significant QTL were obtained for osmotic potential under irrigation (OPi) on chromosome 2H and for WSC100 (water-soluble carbohydrates at full turgor) under drought stress on chromosome 4H. Most of the dESTs identified by Diab *et al.* [226] were "effector" genes, while the study by Tondelli *et al.* [54] represented the first example of an integrated approach for mapping several regulatory candidate genes involved in (cold and) drought response of barley. The authors gave great emphasis to TFs and upstream regulators, rather than to structural genes, following the hypothesis that the trait of interest may be influenced by molecular variation in regulatory genes more than in the structural ones [228]. It could be worth mentioning that of the 12 drought tolerance QTL reported on their consensus map, Tondelli *et al.* [54] found positional association with several regulatory candidate genes on chromosomes 2H, 5H, and 7H, among which *HvABI5* on chromosome 5H, and with drought-induced effector genes on chromosomes 5H and 6H. More recently, Chen *et al.* [229] conducted drought resistance QTL mapping on 134 F4 families derived from a cross of two wild barley (*H. vulgare* spp. *spontaneum*) lines in moderate drought. In agreement with previous studies on cultivated barley, three phQTL for relative water content were identified on chromosomes 1H, 2H, and 6H, underscoring the importance of the 1H and 6H genomic regions for the maintenance of plant water status. In particular, the RWC-phQTL on chromosome 1H is coincident with that identified by Teulat *et al.* [230], while the RWC-phQTL on chromosome 6H was coincident with two QTL found under field conditions for RWC and carbon isotope discrimination, by Teulat *et al.* [227, 231]. A QTL for seedling regrowth rate (REG) identified by Chen *et al.* [229] on chromosome 4H is coincident with a QTL for RWC under Mediterranean field conditions [231]. A genomic region on chromosome 6H [229] corresponds to a previously identified QTL that control leaf osmotic potential and osmotic potential at full turgor, scored under Mediterranean field conditions [231]. Finally, Chen *et al.* [229] found a genomic region on chromosome 2H, near AFLP marker W3555-113, with QTL effects on leaf relative elongation rate (RER), second leaf length (L2L), and root length (RLE). This region might match both a QTL effect for RWC under Mediterranean field conditions [231] and a QTL for salt tolerance reviewed by Cattivelli *et al.* [180]. As was briefly summarized here, grain yield has been the subject of numerous QTL studies in barley, but most of these have been conducted in relatively high yielding environments. Comadran *et al.* [188]

assembled a population of 192 genotypes that represented landraces and old and contemporary cultivars from Europe and WANA (West Asia and North Africa). This population was grown in contrasting rain-fed and irrigated sites through the Mediterranean countries. By LD mapping, QTL exhibiting consistency across environments were detected in Bins 4, 6, 6, and 7 of barley chromosomes 3H, 4H, 5H, and 7H, respectively. The authors were able to detect between two and five significant QTL in severely stressed environments with field mean yields lower than 2 ton ha^{-1}. As one of the most interesting findings, one of the most consistent genomic regions was located in Bin 7 of chromosome 7H, where four out of the five significant associations came from the four Jordanian sites with mean grain yield ranging from 0.3 to 1.2 ton ha^{-1}.

34.2.5.2.3 **phQTL for Salt Tolerance** Barley is one of the most salinity-tolerant cereals, and barley cultivars display different levels of tolerance toward salinity [141]. As outlined in Section 34.1, salt stress has at least two components, an osmotic one and an ionic one, due to sodium (Na). Generally, barley plants are more sensitive to salinity at germination and young seedling stage, while they exhibit increased tolerance with age. No correlation was observed between salt tolerance at seedling and salt tolerance at germination stage in barley, suggesting stage-specific mechanisms [232]. Salt tolerance of barley has been of interest for a long time and has resulted in a considerable body of data from studies using physiological [233], genetic [234], and cytogenetic approaches [235]. The first evidences of salinity tolerance QTL date back to the second half of the last decade, and they are related to sodium tolerance at germination stage [232]. These authors reported that salinity QTL at the seedling stage were located on chromosomes 1H, 2H, 5H, and 6H in the DH lines of "Steptoe" × "Morex" and on chromosome 5H in the DH lines of "Harrington" × "TR 306." More recently, in the work of Witzel *et al.* [236], a QTL analysis of the Oregon Wolfe Barley ("DOM" × "REC") population germinating at different salt concentrations led to the identification of two chromosome regions on 5H and one on 7H associated with salt stress response. The same population was also used to identify via 2D-GE six protein spots with a differential abundance between the tolerant and the sensitive lines. Based on a dense barley transcript map [55], two of the more expressed proteins, Hsp 70 (heat shock protein 70) and Glc/RibDH (glucose/ribitol dehydrogenase), were assigned to a genetic location and colocalized with the identified QTL on chromosome 5H [236]. Other genomic experiments like those of transcriptional profiling in response to salt stress by Ozturk *et al.* [141], Ueda *et al.* [147], and Walia *et al.* [14, 148] allowed to find a significant selection of transcription factors and other candidate genes for salinity tolerance (see Section 34.2.4.2). These are a reservoir of coding sequences for explaining with genetical and genomic evidences each salt tolerance phQTL. In the near future, they could be used to improve resilience to an abiotic stress relatively easy to phenotype, on large numbers of plants, if compared to the others described.

34.2.5.2.4 **phQTL for Nutrient and Metal Stress Tolerance** Increasing the efficiency with which crops utilize nutrients, in particular N and P, represents an urgent priority

for ensuring a cost-effective and sustainable agriculture for the future. As regard nitrogen, nitrogen use efficiency (NUE) is a synthetic index that indicates the biomass nitrogen yield per unit of nitrogen consumption, calculated on the basis of a complete nitrogen balance. In case of cereals, worldwide NUE was estimated approximately 33%, and might be estimated by the equation NUE = (total cereal N removed – N coming from the soil + N deposited in the rainfall)/(fertilizer N applied to cereals) [237]. As a synthetic index, it depends on several known agronomic traits such as the extent of root–soil association, the root N uptake, the N metabolism, and the N translocation and remobilization from vegetative tissues to the grain. Although under high environmental influence, and depending upon agronomical practices, attempts to uncover the genomic regions associated with both NUE and nitrogen-related traits in maize and other crops have been made. QTL for tolerance to low N were mapped in barley. Several QTL for nitrogen-related traits, namely, QTL associated with nitrogen uptake, storage, and remobilization, were located practically on all chromosomes, although NUE was not calculated. Chromosomes 3H and 6H were especially important, for their QTL, alleles associated with inefficient N remobilization were also related to depressed yield [238]. No map comparisons between QTL and candidate genes were performed. In maize were reported a high number of QTL mapping studies for nitrogen-related traits (see, for example, Coque and Gallais [239]). A number of genes that encode enzymes involved in N and C metabolism were associated by mapping to QTL for vegetative development and for grain yield and its components [240]. These included genes for Gln synthetase (GS): Suc-P synthase, Suc synthase, and invertase (b-fructofuranosidase). The most notable outcomes of these studies were the colocation of a major grain yield QTL on maize chromosome 5 with the gene encoding cytosolic GS (*gln4* locus) and the correlation between the expression levels of the *gln4* alleles and the contributions of the respective QTL alleles at this locus.

Because of the low mobility of P in the soil, extensive QTL mapping work has been carried out to investigate the effects of root architecture on P uptake and use efficiency, although not in barley. A common feature of these studies was the finding that the QTL alleles for high P efficiency were associated with greater root surface area due to an increase in either root mass or root hair density. Interestingly, in rice, marker-assisted backcross breeding of the positive allele at *Pup1*, a major QTL on chromosome 12 for P uptake efficiency, provided introgressed lines with a three- to fourfold increase in P uptake [241].

The pattern of inheritance of tolerance to excess Al, phenotypically identified as better plant growth and biomass accumulation, has been explored in barley by different genetic approaches, including phQTL mapping, leading to the identification of a genomic region of highest importance on chromosome 4H. In this region, four major genes were identified and named *Alp*, *Pht*, *Alt*, and *Alp3*, outlining that this trait, different from other resistances to abiotic stresses, is not properly a quantitative trait in barley. The locus *Alp* conferring Al tolerance to cultivar "Dayton" was located on the long arm of chromosome 4H by crossing "Dayton" with the trisomic line "Shin Ebisu 16" [242]. The same chromosomal location (4H) of other proposed Al tolerance loci was confirmed including *Alt* from "WB229" by means of

amplified fragment length polymorphism (AFLP), SSR, and analysis of wheat–barley chromosome addition lines [243]. At present, the best candidate gene to explain the tolerant phenotype remains *Alp* gene that codes for a protein that belongs to the MATE family [244]. Furukawa *et al.* [163] reported an aluminum-activated citrate transporter gene in barley, *HvAACT1*, responsible for Al tolerance that also belongs to the MATE protein family. Recently, minor QTL effects for Al tolerance have been reported [245, 246], indicating the existence of a multigenic, quantitatively inherited component of excess aluminum tolerance in this cereal, besides the major gene effect (s). Particularly, Raman *et al.* [245] identified several phenotypic QTL for root elongation under excess Al stress on 3H, 4H, 5H, and 6H chromosomes in an F2 population from "Ohichi" × "F6ant28B48-16," whereas Navakode *et al.* [246] tested the Oregon Wolfe Barley (OWB) population and detected minor QTL effects located on 2H, 3H, and 4H chromosomes.

Boron (B) is an essential micronutrient for higher plants, but high levels of boron in the soil can seriously diminish grain yield in cereal crops. The stress damage consists in affecting root growth, thus restricting water extraction from the subsoil. Amelioration of the high B levels in soils is not always feasible; therefore, assisted breeding for B tolerance in barley has moved forward since the work of Jefferies *et al.* [9], who identified both a range of phenotypic assays for assessing different physiological aspects of boron tolerance and molecular markers for tagging loci for the trait. The source of alleles conferring B tolerance is "Sahara," an unadapted six-rowed barley landrace of Algerian origin. Jefferies *et al.* [9] identified four significant phQTL involved in different phenotypic aspects of B tolerance. A region on chromosome 2H was associated with leaf symptom expression, a 3H QTL influenced root growth suppression by boron toxicity, a region on 4H influenced boron uptake, root growth response, dry matter production, and leaf symptom expression, and a region on 6H was associated with B uptake. After validation through a backcross approach, the authors concluded that the chromosome 2H locus is the most important in controlling leaf symptom expression, while the chromosome 4H locus appears to be the most important in controlling B uptake, root length response, and dry matter production. A few years later, physiological studies assessed that a high level of tolerance to B toxicity is mediated by extrusion of B from the root [247], resulting in lower concentrations in roots and shoots. Based on the result obtained by Jefferies *et al.* [9], two of the four boron toxicity tolerance loci have been cloned so far. Sutton *et al.* [165] followed a map-based approach to isolate a CG for the 4H boron tolerance QTL. Using a population representing 6720 meioses and gene colinearity with the syntenic region on rice chromosome 3, they identified the *Bot1* gene, a putative integral transmembrane B transporter with similarity to bicarbonate transporters in animals [165]. Recently, Schnurbusch *et al.* [248] reported the cloning of a gene underlying the chromosome 6H B toxicity tolerance QTL. The gene, named *HvNIP2;1*, encodes an acquaporin from the nodulin-26-like intrinsic protein (NIP) subfamily, which corresponds to the previously described gene *HvLsi1* (low silicon 1), a silicon influx transporter in barley and rice [249]. Interestingly, the observed differences in mRNA levels of *HvNIP2;1* in "Clipper" and "Sahara" might be explained by a repeat insertion of ∼2 kb upstream of the translation codon in

"Sahara." The authors also hypothesized that such repeat insertion might be responsible for altered tissue- or cell-specific expression of *HvNIP2;1* in the root. Together, the findings of Sutton *et al.* [165] and Schnurbusch *et al.* [248] suggest a mechanism for barley tolerance to high soil B in which the reduced expression of *HvNIP2;1* limits B uptake and the increased expression of *Bot1* removes B from roots and sensitive tissues.

34.2.5.2.5 **phQTL for Waterlogging Stress Tolerance** Waterlogging conditions substantially cause to barley plant a hypoxic stress [5]. Barley germplasm showed significant differences in waterlogging tolerance [250], and locally adapted landraces could be major sources of tolerance to be used for breeding. However, studies of tolerance to flooding, namely, waterlogging, have been performed under notably different conditions, from paddy field trials to laboratory tubes, and at different growing stages. In turn, Stanca *et al.* [5] underlined how the genotypes resistant to water sensitivity of germinating seeds were different from those tolerant to flooding after germination. Waterlogging tolerance is, therefore, a complex trait, with different tolerant phenotypes to different stress conditions, from seed to adult plant developmental stages. Moreover, such a trait is affected by several additional (confounding) environmental factors, such as temperature, nutrient availability, soil type and subtopography, and possible presence of concurrent biotic stresses. Besides, breeding for flooding tolerance is difficult because of low heritability of the trait [251]. Little progress has been made since the last decade in mapping phQTL controlling waterlogging tolerance in barley. In these studies, many traits including the ability to produce high seed yield in flooded fields, leaf chlorosis, plant height, root biomass, and shoot biomass have been used as determinants of flooding tolerance. Among all the different criteria, leaf chlorosis after waterlogging has been one of the major indices used by researchers in different crops such as wheat [252], soybean [253], and barley. Li *et al.* [254] carried out a QTL analysis in two doubled haploid populations measuring the leaf chlorosis trait and found at least seven QTL for waterlogging tolerance in the barley genome. Three QTL on chromosomes 1H, 3H, and 7H were very stable and were validated under different stress durations, in different environments, and in different populations. Some of the detected QTL affected differently scored traits; for example, the QTL on chromosome 4H not only reduced leaf chlorosis but also increased plant biomass under waterlogging stress, whereas other QTL alleles such as those on chromosomes 2H and 5H both alleviated leaf chlorosis and improved plant survival.

Zhou [255] accurately phenotyped leaf chlorosis at early stages of waterlogging and plant healthiness including survival at late stages of the treatment for QTL mapping. Curiously, he found that both the significance and the extent of phenotypic effects of the QTL controlling waterlogging tolerance varied at different stages of treatment. At the very early stage (2 weeks) of waterlogging, only three minor QTL were identified, explaining a less than 30% of the phenotypic variation. As the duration of waterlogging increased, higher significant QTL were identified and these explained more than 50% of the variation. Some of the QTL found at different stages of waterlogging were different, but the two major QTL on chromosomes 2H and 4H identified at the

final stage of the stress treatment were coincident with two regions found at the early stages of the treatment.

34.3 The Contribution of other Omics Approaches

During the past few years, we have been part of the exciting progress the "omic sciences" have made in the dissection of complex traits in plants. Like genomics, proteomics and integrative omics have well demonstrated their potential to shed light on the molecular basis of plant phenotypes in model plant research (above all *Arabidopsis*). Conversely, for many crop species the situation is far less advanced since nucleic acids research has been largely favored to other "omics" research. However, a good deal of omic evidence had accumulated for crops and in particular for barley. The information obtained in such studies, when well established, will be of fundamental importance for (1) studying and modeling barley crop from an integrative systems biology perspective or (2) the identification of useful molecular/phenotypic variants conferring better adaptation to the environment. This section summarizes the contribution of the "other omics" to a comprehensive biological knowledge of barley abiotic stress tolerance.

34.3.1 Proteomics

The proteome can be defined as the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism or a system. In other words, the structure and the functions of the proteome are studied through large-scale experiments by proteomics. Proteomic platforms have been applied to various aspects of cellular processes, not only to the identification of proteins but also to the determination of the protein expression profiles during development and under stress conditions, to the analysis of posttranslational modifications (PTMs), and to the study of protein–protein interactions. Temporal and spatial profiling of protein spots can be used to associate proteins with developmental processes and to group proteins with similar profiles.

Recent improvements in protein analysis methods have made possible the evaluation and identification of many more proteins and to exploit proteomic data in the context of stress response (for a review, see Ref. [256]). The use of a gel-free proteomics system in addition to a traditional two-dimensional gel-based system provides a vast amount of information on proteins expressed, and a detailed analysis of PTMs via mass spectrometry enables the uncovering of key signaling molecules. Moreover, significant results have recently been obtained through focused analyses, till the level of subcellular proteomes. Once technically possible, the tissue microdissection, or the subcellular components with dedicated extraction procedures, additional important information could be obtained by examining at proteomic level the tissues separately or by analyzing specific cellular components or organelles.

Some of the drawbacks encountered in plant proteomic studies relate to the following aspects. Plant tissues are difficult to disrupt due to the rigidity of cell walls, which are made of a complex assembly of polysaccharides. Protein extraction is also complicated by the presence of secondary metabolites, mainly polyphenolic compounds, which can cause protein precipitation, artificial spots, or streaking on two-dimensional gel electrophoresis (2D-GE), and charge heterogeneity [257]. Extremely abundant proteins such as rubisco (that constitutes approximately 30% of proteins in photosyntetically active leaves) can mask the detection of lower abundance proteins by MS, but reliable and robust methods to remove highly abundant proteins from plant tissue samples are still under development. A major challenge faced in the past by shotgun proteomics has been the protein inference problem (i.e., the assignment of identified peptides to the exact proteins from which they were derived). This is either due to the presence of multiple peptide in the same spot after 2D-GE or due to the identification of a set of redundant and homologous peptide sequences that in many cases could represent multiple proteins. Possible solutions to the inference problem have also been published in plant proteomics. For example, Grobei *et al.* [258] applied gene model–protein sequence–protein accession relationships to classify and eliminate ambiguities inherently associated with any shotgun proteomics data set and reported a conservative list of protein identification to seamlessly integrate data from previous transcriptomics studies. Moreover, proteotypic peptides (i.e., peptides that can be observed by MS and uniquely identify a specific protein or a specific isoform of a protein for the chosen targets) can be selected either using software tools to predict their sequences or accessing existing public data depositories [259].

Initially applied to *Arabidopsis*, functional proteomics has been devoted to the high-throughput identification of all of the proteins present in plant cells and/or tissues, in order to generate large-scale proteome data sets. Today, these include, for the plant model species, quantitative proteomics, subcellular proteomics, and various modifications and protein–protein interactions (for a review, see Ref. [260]). The different Web-accessible plant proteome-related databases are summarized on the web site of the proteomics subcommittee of the Multinational Arabidopsis Steering Committee (MASCP, http://www.masc-proteomics.org), under the heading of "Proteomic Databases and Resources." The proteome database is the basic platform from which proteome research addresses specific biological questions. In fact, integrated information about precise high-resolution proteome maps becomes a prerequisite for functional gel-based proteomics based on evaluating the changes in protein expression, and for exact protein identification in mutants and in stress-challenged plants.

Some excellent reviews have been published about the state of proteomic research in the context of crops (see, for example, Refs [256, 261]) and/or some specific crops such as the monocot rice [262] and the dicot soybean [263]. In particular for rice, the crop that has the advantage to be contemporarily a genomic model plant, a systematic proteomic analysis of leaves, roots, and seeds has been done and proteome maps developed by Koller *et al.* [264]. Data have been collected in the Rice Proteome Database, which catalogues rice proteins of different tissues and organelles ([265], http://gene64.dna.affrc.go.jp/RPD/).

The proteomic approaches used in barley so far focused basically on the seed proteome [266], rather than on abiotic stress metabolism. This is because barley is largely grown for malting, and for this purpose the seed development and germination are crucial. Proteome analysis during grain filling, maturation, and germination has been applied to describe in detail the protein changes occurring in the barley grain. Seed tissue subproteomes have also been analyzed, thus giving a spatial–temporal overview of the seed protein profiles. Although representing an investigation of seed proteome on a panel of superior malting quality cultivars and not related to physical stresses, the work of Finnie *et al.* [267] deserves mention because of the approach they followed. Interestingly, the authors showed that some spot variations measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) peptide mass spectrometry were caused by amino acid differences encoded by single-nucleotide polymorphisms (SNPs). Coding SNPs were validated by mass spectrometry, expressed sequence tag, and 2D gel data. This demonstrated how coding SNPs can alter function of affected proteins and may thus represent a link between cultivar traits, proteome, and genome. In addition, this type of analysis can identify not only chromosome locations of transacting regulators of the proteins observed but also structural genes encoding the proteins themselves [268]. Expression of the peroxidase gene on chromosome 7H was reported to be affected by low-temperature and drought stresses [44, 267], and therefore, the presence or absence of the protein in different cultivars might be an indicator of stress tolerance.

34.3.1.1 Proteome Response to Temperature Stress

A wide range of proteomic studies of temperature tress in plants are in progress, using numerous methodologies, species, and stress conditions (for a review, see Ref. [269]). Despite results obtained so far, information on the systemic response to temperature stress is still limited because plant perception and response is often based on factors common to the response to other stresses. It is at least clear that high- and low-temperature stresses cause distinct proteome responses in plant tissues [269]. One of the key factors for heat tolerance is the heavy induction of heat shock proteins by their upstream transcription factors. Low temperature stress response is characterized by significant effects on chloroplast protein components and energy production, ROS detoxification, and accumulation of small osmoprotectant molecules synthesized by enzymes that are under the control of key transcription factor proteins (see Sections 34.2.4 and 34.2.5). Comparison of wheat cultivars with different heat tolerance levels identified low molecular weight (16–17 kDa) HSPs and other metabolic proteins in the wheat grain [270]. A quite comprehensive catalog of heat stress-responsive proteins has been provided for rice, although still not for barley, revealing that the majority of heat-affected proteins were small HSPs [271].

In barley, proteomic analysis of heat stress response has been performed on shoots [272]. Two spring-type cultivars, "Mandolina" (heat stress tolerant) and "Jubilant" (stress susceptible), were subjected to 40 °C for a short-term heat shock (2 h). Analysis of extracted proteins by 2D gel electrophoresis followed by MS/MS

identified several proteins being differentially expressed in both cultivars. Among them, distinct isoforms of several HSPs were found and in particular the expression of *S*-adenosylmethionine synthetase (SAM-S) was found to be upregulated in "Mandolina" compared to "Jubilant." Expression of SAM-S is known to increase under some stress conditions and the authors proposed it as a heat tolerance marker [272]. Two HSPs were unique to the susceptible cultivar, and one protein that dramatically decreased under heat stress was a 23 kDa oxygen-evolving protein of photosystem II, which is important for photosynthesis [272].

Stress duration is a key factor to the process of cold acclimation. In fact, plant response varies according to the length of low-temperature exposure: long-term (days to weeks) responses follow the process of cold acclimation and grant plants with the ability to survive freezing stress, while short-term (seconds to hours) responses are triggered by cold shocks. Moreover, stress intensity also characterizes the response to low temperatures; plants unable to acclimate and withstand freezing, such as rice or tomato, respond only to the above-zero temperature chilling stress. Several attempts have been made to understand the variation in cold response in the rice proteome, and a collection of recent literature can be found in Ref. [269]: mostly changes in the proteome of anther, leaf, and root tissues were investigated, producing a long list of proteins up- and downregulated in response to chilling stress in rice. These ranged from chaperonins and HSP proteins to elongation factors, ribosomal and ribonucleoproteins, from ATPases and ATP synthases, to dehydrogenase and decarboxylase enzymes [269]. On the contrary, for barley a limited or no information is available on proteome variation following exposure to low temperature. This could be in part due to the fact that the metabolic response of barley to cold stress has already been studied in much detail using genomics approaches. And this might suggest a potential limit of proteomic research that when large functional genomics data sets are already available, it does not add a lot to the qualitative knowledge of the metabolic pathways and gene products activated or repressed. However, the level of transcript abundance does not always correlate well with the level of the corresponding proteins, the key players in the cells. Transcription intensity of a gene gives only approximately its level of translation into a protein since abundantly transcribed mRNAs may be degraded rapidly or translated inefficiently, resulting in still lower abundance of protein [273]. Finally, proteomics could show in the future its higher value in identifying what such data sets cannot contain, such as protein–protein interactions, or PTM; as in the case of the barley response to low-temperature stress, where key transcription factors are acting.

34.3.1.2 **Proteome Response to Drought and Salt Stresses**

As we mentioned before, several proteomics studies have been performed on crops exposed to abiotic stresses, in particular rice. Apart from temperature stress, about one-fifth and one-third of the works reviewed by Salekdeh and Komatsu [261] dealt with drought and salt stresses, respectively. Unfortunately, most of the proteomics research on drought and salt tolerance has not been done in barley, where genomics has been the far most preferred approach. To keep proteomic studies relevant to the field situations, where crops may experience several constraints either simultaneously

or at different developmental stages, the speed with which drought stress is applied must be taken into account. For example, it is slower in soils compared to pots that tend to dry out faster. Equally important are the responses associated with severe water deprivation and subsequent rewatering because the metabolic machinery must be prepared to rapidly reinitiate growth upon water entering tissues and cells. In this regard, the proteome analysis of field-grown sugar beet plants performed by Hajheidari *et al.* [274] demonstrated the predominance of proteins related to ROS handling, protein folding, and stability. Another example was obtained in wheat (*T. aestivum* L.) comparing three different spring genotypes cultivated in the field under well-watered and drought conditions [275]. Phenotypically, the overall effect of drought was highly significant in terms of dry matter and grain yield. The authors detected and analyzed about 650 spots on 2D gels and more than 120 proteins with significant change under water deprivation in at least one genotype. It is worth noting that about two-thirds of the 57 proteins identified via MALDI TOF/TOF MS were Thioredoxin (Trx) targets. This is also in accordance with the link between drought and salt stress observed in transcriptional changes of the barley cultivar "Tokak" [141], where Trx played a key role. However, a higher drought tolerance of barley compared to wheat attributed either to specific mechanisms of adaptation to water deprivation or simply to a broader adaptation to the environment has still to be found.

The role of the proteins involved in reactive oxygen species detoxification during salinity stress has been studied by Witzel *et al.* [276]. They analyzed via 2D-GE the root proteome of two well-known genetic mapping parents, cvs. "Steptoe" and "Morex," which possess contrasting levels of salinity tolerance. Proteome maps of plants grown under nonsaline versus saline conditions revealed cultivar-specific and salt stress-responsive protein expression. In total 26 out of 39 2D-GE spots were identified via MS, and 2 proteins involved in the glutathione-based detoxification of ROS showed higher abundance in the tolerant genotype ("Morex"), while proteins involved in iron uptake were expressed at a higher level in the sensitive one ("Steptoe"). Of the proposed targets for barley salinity tolerance improvement an induced protein, which was more abundant in cv. "Morex," was identified as a putative target for Txr-mediated reduction [276]. In a follow-up experiment, Witzel *et al.* [236] combined proteome maps' comparison with a CG approach. As reported in Section 34.2.5.2, the authors mapped two major QT loci of salinity tolerance at germination stage in the Oregon Wolfe Barley "*DOM*" × "*REC*" population, one on chromosome 5H and one on chromosome 7H [236] (see also Figure 34.1). A search for candidates was then based on 2D-GE proteomic profiling of the mature grain of a subset of OWB lines, and six protein spots showing differential abundance between tolerant and sensitive genotypes were identified.

34.3.1.3 **Proteome Response to Metal and Nutrient Stress**

Proteomic methods based on classical 2D-GE provide a generally good visual output for protein profiling and comparative mapping of expressed proteins among biological samples. They have been extensively applied in various tissues and crop species, for example, soybean, in response to salt stress [277], rice root proteome in response to cold [278], and the seed proteome of wheat [279] and barley [266]. Second-

generation proteomics technologies have also been developed and, among them, the isobaric tags for relative and absolute quantitation (iTRAQ) technique represents an interesting system for quantitative proteomics after chemical labeling using stable isotopes. iTRAQ has found particular application in the study of crop proteomes against heavy metal stress at both plant [280] and subcellular [281] levels. For example, Patterson *et al.* [280] used the iTRAQ peptide tagging system to investigate the proteomic response to boron (B) excess in hydroponic growth solution of B-tolerant and B-intolerant barley cultivars. In particular, the authors found increased activities of three enzymes involved in siderophore production (related to iron deficiency) and suggested a potential link between iron, B, and the siderophore hydroxymugineic acid under B stress [280]. More recently, the vacuolar proteome of mesophyll cells of barley leaves was investigated in response to cadmium (Cd) stress [281]. This study pointed out that a c-tonoplast intrinsic protein (c-TIP) may have a specific function in Cd transport into the vacuole and play an important role in Cd^{2+} detoxification of barley leaf mesophyll cells. The proteomic study provided valuable insight into heavy metal stress biology of barley. However, it is quite a general opinion that still a deal of work remains to be done in the enormous and nebulous field of heavy metal toxicity in plants [282]. This includes identification of protein networks responsible for several processes inside the same response: translocation, biotransformation, and sequestration-related activities. Analyses of subcellular proteome, posttranslationally modified proteins, and/or redox proteome is expected to help in this big challenge [283].

Mineral nutrient deficiency is a factor severely influencing plant biomass production in many cultivation areas. Some interesting examples aimed to assess differences in nitrogen utilization by small-grain cereals have been obtained in wheat and rice crops both at the root [284, 285] and the leaf levels [286, 287]. In all cases, 2D-GE and mass spectrometry identification were applied to investigate the nitrogen stress-responsive proteome of widely contrasting cultivars and hundreds of protein spots were reproducibly detected. Among the proteins identified in the leaves were enzymes involved in carbon fixation and energy production, whereas nitrate reductase and glutamate dehydrogenase were the most influenced root-specific proteins. However, because a large number of spots with differential expression during the varying experiments/treatments and/or varieties still remained to be identified, a full picture of responses to difference in nitrogen utilization could not yet be made.

Although no examples concerning nitrogen stress as far as we know have been published on barley, we expect that advances in proteomic technologies will be of great help in the near future to clarify the role played by the barley proteome in response to N deficiency.

34.3.1.4 Roles of "Interactome" and Comparative Proteomics

It is increasingly clear that protein–protein interactions play a key role in all metabolic processes, and in particular in response to abiotic stress [261]. The (re)creation of interaction networks from proteomic data (the so-called "interactome") will thus provide novel insights into how cells perceive and transduce stress signals to activate

the appropriate cellular response, which could then be compared with and linked to genomics-inferred gene networks. In this regard, an important application, unique to proteomics research, is the ability to study posttranscriptional modifications, which can affect the activity and binding of a protein and alter its role within the cell. About 300 posttranscriptional modifications have been classified, including methylation, phosphorylation, glycosylation, acetylation, ubiquitylation, and so on. In particular, together with the interactome, the phosphoproteome and "glycomics" (if we would like to call in this way the comprehensive study of the entire complement of sugars) will soon become major areas in proteomics-related research.

Among other considerations with regard to future proteomics research, one deserving attention derives from the fact that as data on proteomic experiments accumulate, comparative analysis could be done. For example, Salekdeh and Komatsu [261] compared 56 abiotic stress proteome studies conducted by different research groups in crops. The authors found that the number of stress-responsive proteins identified diverged considerably from each other and only less than 20 proteins were commonly identified under different stress conditions. This divergence probably derived by the fact that many stress-responsive proteins still remain to be identified or that the sampling of tissues was not comparable between tissues and experiments. Even for similar type of stress, different levels of treatments and sampling times and sample preparation were applied [261]. Thus, at the moment, it is difficult to draw consistent conclusions from comparative crop proteomics studies. Only a higher conformity of proteome analysis between different groups of investigators, as well as the application of standard operation procedures, would facilitate comparability of results in barley as in other plants.

34.3.1.5 **Proteomic and Proteomic-Related Databases**

A number of resources for plant genomics accessible on the Web have appeared during the past two decades. The majority of them provide gateways for accessing comprehensive omics data and/or bioresources for the model plant *Arabidopsis* (e.g., TAIR, AraCyc, etc.). In addition, in recent years, several integrative databases on crop plants have been properly developed worldwide (for a recent review, see Ref. [85]). None of them is entirely dedicated to barley, and many include information on the major species rice and maize, together with sorghum. Within this context, we are going to describe here three interesting examples of integrative databases that, besides those about other crops, include relevant proteomics or proteomics-related information about barley. These are GabiPD [288], MetaCrop [289], and PlantTFDB [290].

GabiPD (http://www.gabipd.org, [288]) is a Web-accessible database developed in the frame of the German initiative for Genome Analysis of the Plant Biological System (Genomanalyse im biologischen System Pflanze, GABI) that allows integration of varied "omics" data types obtained from plant systems. The database includes information from 14 different angiosperm species with *Arabidopsis* as the most widely represented model species, followed by the crop plants, potato and barley. Genomic data comprise mapping information, sequences, and SNP/InDel information. Transcriptomics is represented by a large number of ESTs and corresponding

sequence trace files [288]. The flexible design of GabiPD allows for a high level of data integration, and eases cross-referencing the different data types among each other (e.g., mapping information, sequences, 2D gel images, and protein information). For barley, EST clustering results and corresponding information on a new 27 000 unigene set are accessible and downloadable. Annotated 2D gel electrophoresis images from *Arabidopsis* and *Brassica napus* L. have also been integrated as a type of proteomics data. Finally, the value of GabiPD is also further increased by integration with other general databases such as TAIR and GenBank, as well as by providing cross-links to secondary databases, such as ARAMEMNON (http://aramemnon.botanik.uni-koeln.de/) and PlnTFDB (http://plntfdb.bio.uni-potsdam.de/v3.0/).

MetaCrop (http://metacrop.ipk-gatersleben.de [289]) contains hand-curated information of about 40 major metabolic pathways in various crop plants with special emphasis on the metabolism of agronomically important organs such as seed and tuber. Species of both monocotyledons and dicotyledons are represented. The database offers an overview of fundamental biological processes because it integrates plant genomic (EST contigs) and pathway (pathway maps derived from KEGG) information. In particular for barley, MetaCrop contains information about 36 pathways, 291 enzymatic reactions, 7 transport processes, and 4 compartments derived from 382 references. Reactions incorporate information about involved enzymes (e.g., EC and CAS number), metabolites (e.g., CAS number, molecular weight, and chemical formula), stoichiometry, and detailed location (species, organ, tissue, compartment, and developmental stage). Furthermore, for central metabolism (sucrose breakdown, glycolysis, and TCA cycle), kinetic data are available for the reactions [289].

Several databases providing data sets of genes putatively encoding specific DNA binding transcription factors (TFs) have been developed in many plant species (for a summary, see Ref. [85]). Such focused databases are usually predictions based on computational methods, such as sequence similarity search and/or hidden Markov model search of conserved DNA binding domains. PlantTFDB (http://planttfdb.cbi.pku.edu.cn [290]) represents one such example. It contains information about 22 plant species of both monocots and dicots. In particular for barley, PlantTFDB classifies 778 TFs, which are clustered in 54 families (http://planttfdb.cbi.pku.edu.cn, October 2010).

34.3.2 Epigenomics

As already summarized in this chapter, results obtained since the pregenomics era have provided a basic picture of gene regulatory networks in barley response to abiotic stresses. However, both the genome size and the number of protein coding genes are not always directly related to the complexity of organisms. In many cases, different phenotypes are due to the modification of chromatin structure, which is governed by processes associated with epigenetic regulation. Genome imprinting [291], transcriptional gene silencing (TGS) induced by transgenes [292], and paramutation [293]

are just some of the well-known examples of epigenetic regulation of biological processes in plants. A comprehensive genome-wide catalogue of epigenetic control elements and how these vary across cell states could offer critical insight into the relationships with other "omics" sciences and between genotype, phenotype, and environment [294]. The term "epigenome" has been referred, for a given cell type, to the complete description of chemical changes to DNA and histones as they map onto the genome. The advent of molecular techniques such as chromatin immunoprecipitation (ChIP), and their combination with (1) genomic tiling array hybridization (ChIP-on-chip) and recently (2) massively parallel sequencing (ChIP-seq), has enabled global and whole-genome epigenetic profiling studies. These two main approaches can be viewed as standard technologies used for mapping chromatin state. On the one hand, ChIP-on-chip is ideal for targeted studies of promoters or specific loci, but for working on a genome scale it requires multiple arrays and large quantities of DNA. On the other hand, ChIP-seq is inherently genome-wide, can detect repetitive elements and allele-specific changes, requires few nanograms of DNA, and capitalizes on remarkable recent advances in sequencing throughput [295]. As expected, these approaches found important applications to investigate the mammalian epigenome [296], but during the past few years the study of the *Arabidopsis* epigenome has begun as well [297, 298]. We thus could expect that ChIP-on-chip and ChIP-seq will be applied soon also to the interrogation of the epigenome of many crop species, among them barley.

The process of a reversible control over gene expression and inheritance has also led to the belief that plants may have a flexible short-term strategy of the response to stress. For example, epigenetic regulation is also involved in stress-related memory formation. This retention has been evidenced not only for short duration, as in the case of acclimation to stress conditions, but also for longer times through stable, heritable chromatin modifications. During the past two decades, research on the epigenetic mechanisms involved in abiotic stress resistance has unraveled that regulation of stress-responsive genes often depends on three epigenetic controllers of chromatin remodeling (for a review, see Ref. [299]): (i) histone N-tails modifications; (ii) changes in DNA methylation state; and (iii) action of nonprotein coding RNAs. These will be briefly described in the following sections.

34.3.2.1 **Role of "Histone Code"**

In plants as in animals chromatin remodeling is the dynamic alteration of chemical reversible modifications at specific residues in the histone N-tails. It is operated by several histone modification enzymes, namely, histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases (HMTs), and demethylases (HDMs). Moreover, each histone has variants encoded by different genes. The combination of histone variants and their posttranslational modifications is referred to as "histone code," which determines the chromatin structure and thus regulates transcription. Most research on chromatin remodeling in plants has been carried out in *Arabidopsis* (for a review, see Ref. [300]), where at least 28 different histone modification sites have been identified so far. MS and ChIP analyses showed that enrichments of acetylation, certain phosphorylation, and ubiquitination usually correlate with gene activation,

while biotinylation and sumoylation generally repress gene expression [301, 302]. The consequences of histone methylation lead to highly complex outcomes depending on the N-tail residues that are modified, whether modifications occur in eu- or heterochromatin, and on the organism considered. In *Arabidopsis*, trimethylation of histone H3 Lys4 (H3K4me3) and acetylation of histone H3 Lys9 (H3K9ac) are generally positive markers of gene activation, whereas dimethylation of histone H3 Lys9 (H3K9me2) and trimethylation of histone H3 Lys27 (H3K27me3) are negative markers of transcription [301]. An initial event in the modification process is the recognition of H3K4me3 by effector proteins that contain a protein fold termed plant homeodomain (PHD). This interaction eventually leads to the introduction of other changes in the acetylation and methylation status of H3K4me3-containing promoters [303].

A variety of modifications of histone N-tails occur during stress responses [304, 305], with some combinations that seem to be common to several genes, and others that appear more gene-specific. Once again, the majority of the work has been done in the model plant *Arabidopsis*, highlighting histone stress-specific responses. Exposure to low temperatures progressively decreases H3K27me3 negative modification at the cold-regulated genes *COR15A* and *AtGOLS3*, and this alteration is maintained for up to 3 days after plants are returned to normal conditions [305]. Exposure to drought increases H3K4me3 and H3K9ac positive modifications at the coding region of the response to dehydration *RD29A*, *RD29B*, and *RD20* genes and *At2g20880*, an AP2 transcription factor [304]. At all four genes, these epigenetic marks correlate with enhanced transcription. In addition to these common modifications, enrichment in H3K23ac and H3K27ac has been reported in response to drought in the coding regions of *RD29B*, *RD20*, and *At2g20880*, but not in the coding region of *RD29A*, indicating a gene-specific way of action [304]. Recruitment of HATs to target promoters is mediated by direct or indirect interaction with transcription factors and/or by interaction with acetylated histone Lys residues on the target. This has been shown for the transcriptional adapter (ADA) and the Spt-Ada-Gcn5-acetyltransferase (SAGA) complexes in which AtGCN5 contributes to HAT activity. The recruitment of the ADA/SAGA-like complexes to regulate cold tolerance through chromatin remodeling in target genes has been shown to be mediated by the interaction with AtCBF1 [306].

Reports describing the involvement of the "histone code" and nucleosome occupancy in the regulation of stress-responsive gene expression are still rare in crops, and particularly in barley. One example is the induction of expression of the linker histone variant H1-S after exposure of tomato plants to drought [307]. H1-S appears to be involved in the negative regulation of stomatal conductance. In rice, expression of different members of the HDAC families is differentially regulated by abiotic factors such as cold, osmotic, and salt stress and by plant stress-related hormones such as abscisic acid, jasmonic acid, and salicylic acid (SA) [308]. As another example, both the acetylation and the methylation levels of histone H3K4 were altered on the rice submergence-inducible genes *ADH1* and *PDC1* (namely, alcohol dehydrogenase 1 and pyruvate decarboxylase 1) during a submergence treatment [309]. A simultaneous decrease in H3K4me2 levels and increase in

H3K4me3 together with histone H3 acetylation levels were observed on the 5′- and 3′-coding regions of *ADH1* and *PDC1* in stress conditions. These histone modifications recovered to the initial state after reaeration treatment.

Characterization of HATs in *Arabidopsis* revealed three families of HAT sequences in plants: the GNAT/MYST group (where GNAT is Gcn5-related *N*-acetyltransferases and MYST was named for its founding members: MOZ, Ybf2/Sas3, Sas2, and Tip60), the p300/CREB binding protein (CBP) coactivator family, and the TATA-associated factors – $TAF_{II}250$ family [310]. Twelve *HAT* genes were identified in *Arabidopsis*, of which five belong to the GNAT/MYST group, five to the CBP family, and two to the $TAF_{II}250$ family [310]. Full-length cDNAs encoding the putative protein sequences for the HvELP3 (elongation protein 3 of the GNAT group), HvMYST, and HvGCN5 homologous HATs of barley were recently obtained by Papaefthimiou *et al.* [311]. Expression analysis highlighted significant differences in abundance during different seed developmental stages and between two barley cultivars with different seed size. Application of exogenous ABA resulted in induced expression of *HAT* genes in barley seedlings, with *HvELP3* as the most affected one [311].

Eukaryotic HDACc are grouped into three major families based on their primary homology to the yeast HDACs: (1) the histone deacetylase HDA1 family; (2) the silent information regulator 2 (SIR2) family; and (3) the plant-specific family of histone deacetylase HD2 [310]. cDNA sequences encoding members of the HDA1 family and representing all four classes (I–IV) of the family were isolated and characterized in barley by Demetriou *et al.* [312]. Expression analysis demonstrated that they were expressed in all tissues and seed developmental stages. Differences in transcript abundance both in vegetative and in reproductive tissues were observed among the different genes suggesting functional diversification of the HDA1 members. Differential expression was also evidenced for some of the *HDA1* genes in two cultivars ("Caresse" and "Kos") differing for various traits, such as seed size and resistance to stress [312]. Sequence mapping and expression analysis of two full-length HD2 cDNAs from barley has also been obtained [313]. Both *HD2* genes were found to respond to treatments with ABA, JA, and SA, implying an association of these genes with plant resistance to biotic and abiotic stress.

PcG (Polycomb group) protein complexes methylate nucleosomal histone tails at specific sites, ultimately leading to chromatin compaction and gene silencing. Different PcG complex variants operating during different developmental stages have been described in plants [314]. Four barley *PcG* gene homologues, namely, fertilization-independent endosperm *HvFIE*, enhancer of zeste *HvE(Z)*, suppressor of zeste *HvSu(z)12a*, and *HvSu(z)12b* were recently identified and structurally and phylogenetically characterized [315]. Expression analysis of the barley *PcG* genes revealed significant differences in gene expression among tissues and seed developmental stages and between barley cultivars with varying seed size. Furthermore, *HvFIE* and *HvE(Z)* gene expression was responsive to the abiotic stress-related hormone ABA [315]. Interestingly, transgenic *Arabidopsis* lines cosuppressed for the musashi-1 *MSI1* gene, encoding a subunit of PcG protein complexes and chromatin assembly factor 1, showed increased drought stress tolerance phenotype that was most likely due to the increased expression of many ABA-responsive genes [316].

Finally, the ChIP assay demonstrated that the drought-inducible *RD20* gene is a direct target of MSI1.

34.3.2.2 **Role of DNA Methylation**

DNA methylation is a ubiquitous mechanism of heritable epigenetic modification that occurs, in both plants and animals, on the 5′ position of cytosine. As in the case of histone modifications, cytosine methylation is involved in dynamic regulation of gene transcription in response to developmental and environmental cues. Cytosine methylation may lead to epigenetic memory in the short and medium term through the formation of stable epialleles that are heritable across generations. Heterochromatin, repetitive sequences, and transposons are rich in methylated cytosine. In plants, 5-methylcytosine accounts for as high as 30% of the total cytosine content, and such epigenetic mark can be found at both symmetric (CpG and CpNpG) and asymmetric (CpHpH) sites (where N is any of the four DNA bases and H is A, C, or T). Three main DNA methyltransferase enzymes transfer and covalently attach methyl groups to cytosine: domain rearranged methyltransferase DRM2/DRM3, responsible for *de novo* DNA methylation in all contexts, maintenance DNA methyltransferase MET1, responsible for maintenance of CpG methylation, and chromodomain-containing methytransferase CMT3 responsible for maintenance of methylation of both asymmetric and CpNpG sites. Both the DRM2 and MET1 proteins share significant homology to the mammalian DNA methyltransferases DNMT3 and DNMT1, respectively, whereas the CMT3 protein is unique to the plant kingdom [317, 318].

While the biological roles of maintenance and *de novo* methyltransferases have been extensively studied in *Arabidopsis*, several studies in other crop species is beginning to unfold that stresses can induce changes in gene expression through cytosine hypo- or hypermethylation. Barley orthologues of the genes of three classes of DNA methyltransferase enzymes can be found in public sequence repository databases such as the TIGR Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/). However, at present, no experimental results about the effect of abiotic stresses on them are available. The only study concerning the methylation cycle and its function in barley has been reported for the endosperm development [319]. Using a custom-made high-density macro array of 12 000 cDNA sequences expressed in developing grains and including major enzymes of the methylation cycle, the authors showed that the expression of genes encoding for the storage proteins prolamins was repressed by CpG hypermethylation.

In tobacco, DNA methylation is reduced in the coding region compared to the promoter region of a glycerophosphodiesterase-like gene *NtGPDL* after aluminum, salt, and cold stress treatments, and its reduction was correlated with the more abundant expression of NtGPDL protein [320]. Likewise, maize seedlings exposed to low temperature showed a genome-wide demethylation in root tissues [321]. In particular, a 1.8 kb fragment (ZmMI1) containing a part of the coding region of a putative protein and a part of a retrotransposon-like sequence was demethylated and transcribed only under cold stress. Even after 7 days of recovery, the methylation of the cold-stressed plants was not restored to the initial levels [321]. On the other hand,

osmotic stresses induced transient DNA hypermethylation in two heterochromatic loci in tobacco cell suspension culture [322] and drought stress induced DNA hypermethylation in pea [323].

As we mentioned briefly, histone modifications and DNA methylation might confer within-generational and transgenerational stress memory to the plant [305]. Specific histone modification-dependent pathways appear to mediate methylation of about two-thirds of the methylated loci in the *Arabidopsis* genome [299]. Thus, dynamic histone modification marks could be converted into DNA methylation marks, which are often more stable. However, large-scale analyses using ChIP-seq or ChIP-chip methods are necessary to increase our understanding of the phenomenon of stress memory and to understand whether such epigenetic changes might have an adaptive advantage for stress tolerance.

34.3.2.3 **Role of Nonprotein-Coding RNAs**

With the availability of complete genome sequences in many model plant species (like *Arabidopsis* and *Brachypodium*), the microarray technology has been particularly helpful in the design of platforms able to access and assess dynamic changes in transcriptomes, including short nonprotein coding RNA species. This is worth noting if we consider that differential regulation of expression, splice variations, and noncoding RNA have been proposed as the molecular mechanisms that can explain the huge phenotypic diversity generated in complex organisms using "only" $<$25 000 predicted genes per genome [324]. In particular, nonprotein-coding RNAs might be more abundant in the transcriptome than previously thought, and exerting a more important regulatory function through mechanisms such as antisense RNA or siRNA. For example, the whole-genome transcriptomes of *Arabidopsis* were analyzed with Affymetrix tiling arrays after exposure of plants to abiotic stresses such as dehydration, cold, heat, high-salinity, and osmotic stresses, as well as ABA treatment [325, 326]. Results revealed that thousands of transcripts from unannotated nonprotein-coding regions were up- or downregulated by these stresses. It was also estimated that about 80% of previously unannotated and upregulated transcripts in functional genomics experiments could arise from antisense strands of sense transcripts [325], and thus can influence whole-genome expression. Such high frequency could thus be expected for those crops whose genome sequence has already been unlocked, and in the near future also for barley.

It is now clear that a complex system of multistep regulation controls the various modifications that the mRNA goes through in the cell. For example, RNA helicases and alternative splicing factors, which are implicated in mRNA processing and metabolism, have a close-link with response to abiotic stresses [327]. A related key topic in gene regulatory systems in stress responses is the regulation of gene expression by small RNAs, for which plants have presumably developed unique features compared to animals (for a review, see Ref. [327]). Small noncoding RNAs 21–24 nt in length, double-stranded, namely, microRNAs (miRNAs) and small interfering RNAs (siRNAs), are ubiquitous repressors of gene expression. MicroRNAs are derived from single-stranded primary miRNA (pri-miRNA) transcripts synthesized from *MIR* genes. The pri-miRNA transcript forms a secondary structure

of an imperfectly paired hairpin, which is cleaved by a ribonuclease-like enzyme called dicer-like-1.

siRNAs are derived from long double-stranded RNAs of endogenous origin (i.e., miRNA-directed cleavage products of noncoding single-stranded RNAs, mRNA transcribed from *cis*-antisense gene pairs, mRNAs of heterochromatin, DNA repeats, and transposons) or exogenous origin (as a consequence of transgenes and viral pathogen infection). The process of gene downregulation by small RNAs can be further modulated if we assume that different mRNAs with weak target sites for miRNAs could effectively sequester a miRNA from its *bona fide* targets. Such an idea, put forward recently by Seitz [328], could explain how an increased number of transcripts susceptible to recognition by a given miRNA (present under a certain condition) can, in principle, counteract the accumulation of a particular miRNA, thus allowing the expression of a silenced mRNA.

Small RNA molecules are active players in the molecular response of plants to abiotic stresses, as they participate in the regulation of different pathways coping with environmental stress conditions (for a review, see Ref. [329]). Abiotic stresses not only induce the accumulation of novel antisense overlapping transcripts [325] but also lead to transcript production from transposons or pseudo-genes, which are another source of siRNAs [326]. A clear example of an endogenous siRNA involved in abiotic stress response is given by the nat-siRNA P5CDH–SRO5 [330]. Produced from two overlapping mRNAs, for D1-pyrroline-5-carboxylate dehydrogenase (P5CDH) and Similar to Rcd One 5 (SRO5), the P5CDH–SRO5 was shown to downregulate the expression of P5CDH through mRNA cleavage, leading to decreased proline degradation, enhanced proline accumulation, and salt stress tolerance. However, such regulatory system was shown to function only in *Arabidopsis* and not in other plants [330].

In recent years, large-scale "omics" screens have been applied to clarify the involvement of miRNAs in stress response. For example, Zhao *et al.* [331] carried out a global survey of miRNAs under drought stress in *O. sativa* by a microarray approach. The authors found that several miRNA profiles changed under stress, and focusing on the potential upstream regulatory sequences of the *miR-169g* (which showed significant upregulation), two adjacent dehydration-responsive elements were found. This suggested for MIR-169 a direct involvement in the regulation of the CBF transcription factors [331]. Again, in rice, massive parallel sequencing of small RNA libraries from control and stress-treated plants has allowed the identification of novel miRNAs. Comparing control, salt-, or drought-treated small RNA libraries, Sunkar *et al.* [332] identified 23 nonconserved miRNAs and proposed an additional 40 candidates; only 6 of the identified miRNAs were in common to other monocots [332].

Extensive studies for the identification and characterization of miRNAs are not yet available on barley with the exception of the work of Colaiacovo *et al.* [333]. Through a bioinformatic approach, the authors used the features of previously known plant miRNAs, accessed the public miRBase online catalog (http://www.mirbase.org), and systematically searched for barley miRNA homologues and targets in the publicly available ESTs database. In total, 156 miRNA mature sequences from 50 different

families significantly matched at least one EST sequence in barley, with miRNAs putatively orthologous to those of *Triticum* clearly overrepresented. Many previously known and several putatively new miRNA/target pairs were identified. When grouped into functional categories, the predicted miRNA targets allowed their classification in regulators of biological processes such as development and response to biotic and abiotic stress, with most of the target molecular functions related to regulation of transcription. The sequence of candidate *MIR* genes was also investigated and putative polymorphisms (SNPs/indels) were found, both in functional regions of miRNAs (mature sequence) and at miRNA target sites [333]. Such *MIR* genes could thus represent an interesting source for the future identification of functional genetic variability in the species.

Finally, it is worth mentioning here that small RNAs not only cause posttranscriptional gene silencing by cleavage, or translational repression of complementary target mRNAs, but they are also involved in transcriptional gene silencing. In fact, small RNA profiles revealed a direct correlation between the ability of genomic sequences to produce siRNAs and DNA methylation, with siRNAs supposedly involved in the methylation of at least one-third of methylated loci in *Arabidopsis* [297]. This would happen through a process called RNA-directed DNA methylation (RdDM). Specific histone deacetylases (HDACs) could represent a crucial point for the connection of small RNAs with DNA methylation and histone N-tail modification. It is the case, for example, of HDA6, which has been found involved in TGS [334] and RNA-directed DNA methylation (RdDM) [335].

34.3.3 Metabolomics

The metabolome could be defined as the "quantitative complement of all the low molecular weight molecules present in cells in a particular physiological or developmental state" (for a review, see Ref. [336]). Owing to the complex nature of compounds produced in each plant species, the plant metabolome represents an enormous chemical diversity [337]. Therefore, plant metabolomics is not only a great analytical challenge but also of great importance for plant biology since it is the final product of genes through proteins and through all their reciprocal interactions. Theoretically, metabolomic knowledge enables to explain in detail plant cellular systems and thus permits the researchers to project molecular breeding approaches in order to improve crop productivity of food, pharmaceuticals, biomaterials, and biomass for energy. Last but not the least, complete metabolic profiles can contribute to the understanding of the cellular system in response to changes in the environment. Moreover, genetic variations and changes in metabolite accumulation profiles can be treated as chemical phenotypes to identify loci and genes relevant for a specific metabolic pathway. A summary of metabolic profiling studies performed in plants and their related databases can be found in Ref. [85]. Two interesting examples deserve mention here: the Golm Metabolome Database – GMD (http://gmd.mpimp-golm.mpg.de [338]) and the MS/MS spectral tag – MS2T libraries (http://prime.psc.riken.jp/lcms/ms2tview/ms2tview.html [339]). Through GMD, users have public access to

more than 25 000 mass spectra libraries, metabolite profiling experiments, and related tools, while browsing MS2T researchers have open access to collections of phytochemical LC-MS2 spectra represented by 1 633 639 records from *Arabidopsis*, rice, wheat, and soybean. Containing wide-ranging data acquired from several "omics" research works, the mentioned databases represent crucial information resources and repositories not only for present plant metabolomics but also for further integration of metabolic profiles [85].

Given the large number of studies on the metabolic response of plants to abiotic stress that are accessible through the above-mentioned databases (for a review, see Ref. [340]), here we will mention briefly some of the more relevant. For example, metabolite profiling was used to understand the dynamics of the *Arabidopsis* metabolome in response to low-temperature stress. The metabolome was extensively reconfigured not only in plants overexpressing CBF3, as expected from the prominent role for the CBF cold response pathway in the hardening process [341], but also in plants with natural variation of freezing tolerance [342]. Basically, these data identified that enhanced freezing tolerance is associated with the downregulation of photosynthesis and hormonal responses and the induction of flavonoid metabolism, and provided evidence for naturally increased nonacclimated freezing tolerance due to the constitutive activation of the CBFs pathway. In the chilling-sensitive species rice, Morsy *et al.* [343] obtained a picture of the modification of oxidative products and antioxidative enzymes in combination with the carbohydrate metabolism and indicated a more direct involvement of the ROS-scavenging system in the tolerance process. Sanchez *et al.* [344] reviewed a selection of publications in the high-salinity stress field and by using GC time-of-flight MS profiles of polar fractions from the plant models, *Arabidopsis*, *Lotus japonicus* L., and rice demonstrated the power of metabolite profiling of conserved and divergent metabolic responses among these three species. The authors concluded that a change in the balance between amino acids and organic acids might be a conserved metabolic response of plants to salt stress [344]. Finally, one of the very few examples of application of metabolome profiling in barley in response to abiotic stress is represented by Roessner *et al.* [345]. The authors investigated via GC-MS the metabolite profiles in root and leaf tissues of an intolerant, commercial cv. "Clipper" and a B-tolerant Algerian landrace "Sahara." After exposure to elevated B (200 and 1000 μM in hydroponic solution), the number and amplitude of metabolite changes in roots were greater in the former than in the latter. In contrast, leaf metabolites of both cultivars responded only following high B treatment [345].

We have already pointed out that hundreds of genes related to various metabolic processes change their expression profile in response to P deficiency (see Section 34.2.4.2). However, limited information is available for global metabolite changes of P-deficient plants, especially for cereals. In barley, a nice example of nutrient stress metabolomics has been published by Huang *et al.* [346] who profiled polar metabolites from both shoots and roots of plants grown under various low phosphate conditions. Gas chromatography–mass spectrometry showed that P-deficient plants reconfigure their carbohydrate metabolism initially to reduce P consumption and salvage P from small P-containing metabolites when stress is

severe. Since data suggested that barley behaves as a P-inefficient plant in metabolic adaptation to low P environments, the authors proposed that the manipulation of metabolism through a shift in carbohydrate partitioning could provide a logical strategy for improving P efficiency in barley and perhaps in other cereal crops [346].

Combinatorial approaches that integrate metabolome and transcriptome data have elucidated regulatory networks acting in response to environmental stresses. Clear examples are from *Arabidopsis*, in which the metabolome was analyzed using various types of MS after cold and dehydration exposure, and metabolic profiles have been then combined into regulatory networks together with transcriptome data. Maruyama *et al.* [347] coupled microarray analysis of transgenic plants overexpressing genes encoding DREB1A/CBF3 and DREB2A transcription factors with the metabolic pathways that act in response to cold and dehydration. A similar approach was used by Urano *et al.* [348] to reveal ABA-dependent regulatory networks under dehydration stress conditions by integrating chemical phenotypic changes between wild-type *Arabidopsis* and a knockout mutant of the *NCED3* (9-*cis*-epoxycarotenoid dioxygenase) gene.

As already underlined, metabolite profiling data of segregating populations can also be treated as a quantitative trait for QTL mapping (Section 34.2.5.2). The derived mQTL are expected to enable the discovery of key genes involved in metabolic changes and would aid in the identification of genetic associations between metabolic and/or visible phenotypes [349]. This approach has been successfully applied to model plants and crop species such as *Arabidopsis* [199], poplar [350], and tomato [351]. In addition, correlative patterns between metabolic and genomic diversities can be reconstructed coupling data sets of genome-wide variation along with metabolome variation in germplasm collections representing natural variation [352]. Such strategy will have an even easier application in the near future thanks to the ever-increasing availability of high-throughput genotyping methods, including next-generation resequencing.

To our knowledge, none of the strategies of integration of metabolome data with other "omics" described in this section has been applied in barley, so far. However, we could reasonably hope we will soon learn about results from this new-edge research in crops such as barley, by possessing a wealth of genomic tools and data.

34.3.4
Ionomics

The ionome could be defined as the inorganic complement of cellular and organismal systems, and can be thought of as the inorganic subset of the metabolome. The study of changes in the ionome of an organism, through quantitative and simultaneous measurement of elements in response to "physiological stimuli, developmental state, and genetic modifications," constitutes the central ratio of ionomics (for a recent review, see Ref. [353]). Owing to its high-throughput character as a phenotyping platform, ionomic research enables a rapid generation of large data sets depicting the ionome status of many thousands of individuals. This can thus be

applied to the discovery of gene function (functional genomics) and to the assessment of the global physiological status of plants [354]. Among the several laboratory techniques that are available for application in ionomic research, the most exploited ones are those based on mass spectrometry (MS). In particular, inductively coupled plasma spectroscopy, both mass (ICP-MS) and optical emission (ICP-OES), allows for the parallel measurement of dozens of elements. Obstacles in detecting small differences in the ionome have been overcome over the years by improvements in these techniques [355].

Since the shoot ionome in a plant represents its mineral nutrient and trace element content, it is controlled by multiple physiological processes that start in the rhizosphere and end with phloem recycling and evapotranspiration [354]. Changes in any of these processes involved in inorganic ion transport from the soil solution to the shoot can have a potential effect on the different ionomic signatures. Such shoot ionomic signatures could be useful markers of a particular physiological condition they are associated with because the shoot is a much more accessible tissue for profiling than roots. Using a high-throughput elemental profiling and data handling pipeline to rapidly analyze the shoot elemental composition of thousands of *Arabidopsis* plants, Baxter *et al.* [355] identified and used multivariable ionomic signatures to study plant response to reduced Fe or P nutrition.

The implementation of an information management system able to control all aspects of the process is extremely important for any large-scale ionomics project, where hundreds or thousands of samples are analyzed over an extended period of time. Moreover, due to the complex interdisciplinary nature of large-scale ionomics research, large collaborative projects are required involving various principal investigators' laboratories, institutions, and field sites.

The huge amount of information generated by ionomics could be particularly useful in view of the availability of genome-wide knockout collections of many crop plants, of barley in our case, and in view of the development of high-throughput genotyping methodologies. Once identified, candidate genes could be rapidly tested for validation by screening for the ionomic phenotype of interest in various types of sequence-indexed insertion lines carrying a mutant allele of the gene of interest. This will be a way to control elemental composition, a critical issue both for plant growth and development and for nutrition of humans who use plants for food. In addition, one of the possible future applications of ionomics will be linked to the study of crop response to salinity and heavy metal stresses. We also think that this will be of particular interest to the barley community as it is, as mentioned, among the most tolerant among the species of the tribe Triticeae.

Disclosing the genetic architecture behind mineral ion homeostasis in plants is the critical first step toward understanding the biochemical networks that regulate the ionome. In this view, QTL for several ionomic traits could also be identified in various species. In *Arabidopsis*, rice, and maize, they have been mapped by the use of immortalized mapping populations such as DH lines. Traits as phosphate accumulation in seed and shoot [356], shoot cesium accumulation [357], shoot selenate accumulation [358], seed K, Na, Ca, Mg, Fe, Mn, Zn, and P accumulation [359], and sulfate accumulation [360] have been identified in *Arabidopsis*. In rice and maize,

these traits include P, Si, Na, and K accumulation (for a review, see Ref. [354]). In a recent paper, Buescher *et al.* [361] exploited the natural phenotypic variation present among 12 *Arabidopsis* accessions and 3 recombinant inbred line (RIL) populations grown in the presence of Li, Na, Co, Ni, As, Se, Rb, Sr, and Cd at subtoxic concentrations in several environments. Using high-throughput ICP-MS the concentrations of 17 different elements were analyzed and significant variation was detected between the accessions. Over a 100 QTL for elemental accumulation were identified, and interestingly environment alteration showed a strong effect on the correlations between different elements and the QTL controlling elemental accumulation. Once QTL have been identified with ionomics, genomics tools nowadays available for crop and model plants could be applied to locate the genes that underlie these loci responsible for the ionomic phenotypes and thus describe such traits in a novel way, that is, at the molecular level. The majority of the ionomic studies conducted to date have been limited to a small number of species with the exception of the report by Watanabe *et al.* [362] in which the authors conducted a broad survey of 42 elements in 670 plant species collected from 29 locations. In order to be able to fully understand the ionome, research needs to expand to more species including barley. In the near future, when the third-generation sequencing becomes an affordable reality, high-throughput ionomics phenotyping platforms, combined with other omics tools such as transcript profiling, proteomics, and metabolomics, will be critical for the possibility to fill the still existing gap between our knowledge of genotype and that of phenotype.

34.3.5 Phenomics

According to the International Plant Phenomics Network (IPPN, http://www.plant-phenomics.com) phenomics – that is, the physical and biochemical traits of organisms that change in response to genetic and environmental variation – aims to (1) develop, integrate, and provide novel technologies to analyze plant phenotypes; (2) provide quality assurance measures in the technologies used for plant phenomics; (3) identify gene functions and their relationship with environmental cues; (4) analyze the way environments affect plant structure and function; (5) quantify plant performance in specific environments in the laboratory and in the field; (6) develop new concepts on the interaction between plants and their environment (physical, chemical, and biotic); and (7) transfer novel technologies to applications in plant production and for the analysis of natural plant and ecosystem performance. Of particular interest for the future will be the effort of interaction between phenomics and the other "omics" technologies to approach plant performance in the environment in a holistic way both for basic and for applied research.

For different abiotic stress conditions, both the onset and the intensity of the stress applied can be defined and controlled in a clean manner during a phenotyping experiment. However, as far as studies on drought tolerance in plants are concerned, the level and onset of water deficit is more difficult to control and monitor than, for example, low temperature due to the fact that drought is a dynamic process and that a

combination of the available water in the soil and the plant's water status has to be considered. Jones [222] reviewed the various measures of water status used in plant and soil science with special emphasis: (i) for the mechanistic effects of water deficits on plants; (ii) for breeding of drought-tolerant plants; and (iii) for management of irrigation systems. The author came to the important conclusion that the role of water potential has received too much emphasis rather than cell turgor that can be measured in plants. In addition to this is the common observation that too often the molecular studies do not make an appropriate use of the necessary water status measurements for phenotyping [222].

During the last years, besides classical phenotyping methodologies used to estimate stress damage, some nondestructive image and data analysis became a possible alternative screening approach to be applied in both the lab and the field. In fact, traditional methods of measuring growth are time consuming and costly, especially if they should be recorded on many thousand genotypes as it is often necessary in large-scale omics research. Often, they also involve the destructive harvest of plants. The training of specialized personnel is also a prerequisite for precise and accurate phenotyping. Moreover, measurements of dynamic parameters, for example, stomatal conductance or photosynthetic rate carried out at a single developmental stage, and/or on a single leaf, may not be appropriate indicators of the average of the whole plant. Eventually, a variety of nondestructive spectroscopic and imaging techniques are now available to evaluate photosynthetic performance, plant function, and plant chemical composition, which are potentially scalable from the leaf to the canopy level [363]. Image acquisition constitutes only the first step in a complex high-throughput phenotyping process. High-throughput analyses of images and data are also essential for a functional workflow. Image analysis in plant phenotyping can take advantage of existing software, such as MatLab (MathWorks Inc.) or the free ware package ImageJ (http://rsbweb.nih.gov/ij/), and rely on expertise already developed in other areas. However, the employment of experts in image analysis and computer vision, together with efforts to convince the plant science community of the validity of using image-derived measurements as a valuable alternative to more traditional phenotyping methods, is necessary to promote the deployment of these new technologies [364]. The mentioned technologies can be particularly useful in controlled environments for temporal resolution and monitoring plant growth throughout the entire experiment. They can also enable the screening of large numbers of genotypes and the identification of small differences in growth rate or expression of certain traits [365]. However, phenotype validation of the results under real soil conditions and environments is necessary before selecting parents for breeding. As more and more plant genomes are being sequenced, there is no doubt that automated high-throughput or high-precision phenotyping will increasingly remain one of the major bottlenecks in crop research. Recently, in order to facilitate ways of automated phenotyping in the analysis of natural genetic diversity in cereal crops and in crop response to abiotic stresses, plant phenotyping platforms have spread worldwide. The Scanalyzer system developed by the LemnaTech company (http://www.lemnatec.com) since 1998 represents the most striking example in this field. It has found broad application in many research

areas of crop phenomics, ranging from plant growth and development to the investigation of the root system in either plant–plant or plant–microbe interaction. Concerning barley phenomics, two very recently established facilities deserve mention: the IPK Plant Phenomics Facility at Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben (Germany) and the Australian Plant Phenomics Facility (APPF). Particularly relevant is the APPF, an AUSD 50 million project based on two nodes. One is located at CSIRO Plant Industry and ANU in Canberra and one is constructed on the Waite Campus of the University of Adelaide. At the former, new nondestructive phenomics technologies such as high-resolution infra-red imaging have been adapted for in-depth application to individual plants and for use in the field, and at the latter facility a "Plant Accelerator" provides automated, high-throughput, nondestructive imaging of plant populations in controlled environments. The key tools of the Plant Accelerator node are four "smart houses," which contain conveyor systems to deliver potted plants automatically to four imaging stations for high-resolution, multiaspect imaging (both in the visible and the infra-red wavelengths). Finally, from a collaboration between LemnaTech and KeyGene, a greenhouse service operation that combines high-throughput, noninvasive technology with trait interpretation to exploit phenotypic variation is accessible for service (PhenoFab Europe, http://www.phenofab.com).

In conclusion, high-throughput imaging coupled with plant management systems such as those cited above provides a valuable new tool system that allows a large number of measurements and a more precise dissection of plant responses to abiotic stresses into a series of component traits. Unraveling the genetic basis of these dissected traits will be significantly easier to deal with. In fact, trait dissection by means of high-throughput phenotyping could provide a significant new opportunity to understand the genetic bases of plant response to stress and, what is very important, to exploit this knowledge for the improvement of yield under a variety of stress conditions.

34.4
From Omics to Improved Abiotic Stress Tolerance: Present and Future Strategies

Barley environmental plasticity, largely acquired through its history of domestication and selection, allows the present germplasm to be cultivated from the Northern boreal countries up to the margins of the deserts and, of course, in the fertile temperate areas. Such a diffusion of the crop most likely represents, in terms of geographic parallels, the widest environmental adaptation for a cereal crop. Other species are more limited by the environment, both in colder and in dryer lands. Genetic improvement of barley capitalized on its ability to escape abiotic stresses more than to tolerate them, apart from the adaptation to higher soil salinities that could represent a true tolerance. In addition, a limit of this C3 species is the amount of biomass production (both total and relative to the water used) as its wide diffusion is often coupled with low or very low yields. Therefore, for barley, as for other commodity crops, the challenge to feed a growing world population is open. In view

of this scenario, Fedoroff *et al.* [366], urged to radically rethink agriculture for the twenty-first century, focusing the research efforts to obtain both process (technological) and product (breeding) solutions to cope with shortage of chemical inputs and increasingly fluctuating environmental constraints. The heart of such agricultural paradigms is to close the loops of nutrient flows, from microorganisms to plants, animals, and back, in agricultural systems powered and irrigated as much as possible by sunlight and seawater [366].

This chapter has reviewed the "omics" research of tolerance to abiotic stresses in barley. Genomics results have constituted the larger amount of information and knowledge achieved until now; and as underlined several times, the first approaches were directed toward single stresses and single spatiotemporal levels of the response. This is anyway expected when a researcher is facing an unknown and complex phenomenon: simplification allows reduction of unknown and unexpected variables. This notwithstanding, the omics awareness of the molecular response of barley to the most important abiotic stresses has been consistently growing. Two aspects of the success in dissecting the biological problem should deserve mention. The first is the constant advancement of technological platforms. For instance, in little more than 20 years, third-generation sequencing chemistries took the place of polyacrilamide gel sequencing. Without such a development, there would have been no chances to scale up the experiments and the obtained outputs to the global level. The second is the leading role played by the *Arabidopsis* research, "THE model plant" – as named by Street *et al.* [367] – that delivered many advanced results and hypotheses on response pathways, later verified in *H. vulgare*.

If we look at the many studies carried on the model species, we realize that the achieved omics results, in particular genomics, are impressive. We might consider the following series of milestones. After the identification of the first list of stress-induced genes, the regulons of key transcription factors for the active response to temperature and osmotic stress such as DREB1/CBF, DREB2, AREB/ABF, and NAC have been first identified and characterized in *Arabidopsis* and then found with similar structure and responsiveness in the grasses [368]. Genome-wide transcriptomic studies in *Arabidopsis*, available in the AtGenExpress resource, revealed the existence of a molecular crosstalk among the different abiotic stresses [369], more intense between salt and drought, and between abiotic and biotic stresses [370]. The first putative stress sensors as the histidine kinase AtHK1, and the existence of MAPK cascades for signal transduction, have been identified in *Arabidopsis* [18]. Since practically a decade of *Arabidopsis* abiotic stress research has been clearly in the postgenomics era [327], the availability of the complete genome sequence has facilitated access to essential information for all genes, like the gene products and their function, the transcript levels, putative *cis*-regulatory elements, and the presence of alternative splicing patterns. These data have been obtained from comprehensive transcriptome analyses, studies of full-length cDNA collections and T-DNA- or transposon-tagged mutant lines, under the enhancing frame of the genome sequence information [327]. Research in *Arabidopsis* also allowed to characterize a common, "universal," stress transcriptome, modulated by a broad range of stress conditions: cold, osmotic, salinity, wounding, and biotic. The common response

metabolism comprises the ROS signaling, JA-, and other hormone-regulated genes [371, 372]. It has also been possible, at cellular level, to draw a schema of common stress response pathway genes [372]. The discovery of the stress induction of new regulatory systems that depend on small noncoding RNAs [373], as well as of the contribution to stress responses of posttranscriptional regulations, has been achieved in the model plant [327]. Metabolomics studies allowed to uncover the reorganization of *Arabidopsis* metabolic network and to identify specific metabolites induced by a specific regulon; for example, the accumulation of sucrose, raffinose, galactinol, and myo-inositol linked to the *DREB1A* induction [374]. Protein analyses in the model species allowed to uncover the role played by key regulators through ubiquitination and sumoylation [327]. Studies on the role of Ca^{2+}, plant hormones, regulatory mechanisms involving small RNA molecules, chromatin modulation, and genomic DNA modifications have enabled us to recognize that plants have evolved highly sophisticated systems in response to complex abiotic stresses [327]. The increased emission, and a possible role for particular volatile organic compounds (VOCs) in mitigating the effects of the oxidative stress, has been demonstrated in the model plant [375, 376]. Quite recently, Matsui *et al*. [325] applied *Arabidopsis* Affymetrix tiling arrays to study the whole-genome transcriptome under drought, cold, high-salinity, and ABA treatments demonstrating that thousands of transcripts derive from the unannotated "intergenic" genome regions; these transcripts can be differentially induced by abiotic stresses. Moreover, results achieved in *Arabidopsis* revealed that the transcriptional response of root cells to environmental conditions is mediated by a smaller core set of genes that determines the root cell identity [377], demonstrating that differentiated cells in roots respond differently to various abiotic stresses.

On the trail blazed by *Arabidopsis* research, also in barley the large majority of research gave genomics precedence over other omics. Perhaps, the most significant and original contribution to the global knowledge of the response to abiotic stress of barley was due to the achieved genetic results. In many cases, the QTL responsible for tolerance to abiotic stresses were genetically mapped in barley, besides other crop species such as maize or rice. Figure 34.1 shows an updated summary of the "hot" genomic regions in barley for the presence of abiotic stress tolerance QTL. The figure illustrates how such important genomic regions are not randomly distributed through barley genome, rather they are concentrated on specific Bins, for instance, Bins 9 and 10 of chromosome 2H, or Bins 6–12 of chromosome 5H. On the one hand, this amount of information has allowed linking genomics with breeding, on the other hand, such studies sometimes showed, thanks to positional cloning of the QTL, that the types of mutations, genes, and metabolic pathways that cause the QTL effects are not distinct from those underlying Mendelian traits [378]. This should be sufficient to confirm the fascinating Robertson's hypothesis [379] of quantitative traits caused by smaller effect alleles of genes underlying the qualitative traits. Only a set of abiotic stress tolerance QTL, or their most likely candidate genes, have been cloned or identified in barley until now (reviewed in Ref. [378]), as barley still has not fully entered the postgenomics research phase like *Arabidopsis*. When this border will be crossed, it is expected that such an amount of genetic knowledge would allow either

the cloning of a higher number of barley QTL or the identification of candidate genes for important agronomic characteristics of cereals. This in turn could produce results economically far more relevant than those achieved by cloning *Arabidopsis* QTL.

In the next decade, "integration" is supposed to represent a scientific jump in unraveling abiotic stress tolerance. Such a concept could be contained in the integral view versus the differential view of genetics. A modern approach to genetic problems could, in fact, be integral rather than differential, in a sense analogous to the use of integration in mathematics. The integral view could be best defined as the complement to the differential, gene-centric approach. In the integral approach, a phenotype is more properly not due to a simple modification in DNA but due to such a modification buffered by networks of molecular interactions. These include, for example, the role of the detailed cellular structures of the egg cells, inherited together with the genomic information [380]. The successful so-called systems biology should then combine reduction and integration. Systems biology, which could be defined in different ways, emphasizing in turn the role of dynamic modeling or the integration of multidimensional data [381], is one of the future research fields for barley abiotic stress tolerance. The integration of information could mean both the integration of molecular levels, from nucleic acids to proteins and metabolites, and the spatial integration, from single cells to organs [377, 382], individuals, and ecosystems [383, 384]. Temporal integration could mean the study of the long-term stresses in addition to the shock ones [327], which should turn into plant adaptation. Finally, integration could mean combination of environmental stressors rather than single stresses [12, 374], also of abiotic versus biotic ones [371, 372]. This could lead to nonobvious molecular responses [13] and networks.

Among the first examples of integration of levels is the concept of genetical genomics, where global expression profiles are treated genetically in a segregating population [190]; advances in genetical genomics have recently reviewed by Joosen *et al.* [385]. Such an approach showed that local *cis*-acting eQTL, which in the majority of cases result from *cis*-regulatory variation in the genes under differential expression, range from one-third to half of the total number of eQTL, while *trans*-eQTL, which could be explained in variations in major regulators, for example, transcription factors, have been in some cases demonstrated to correspond to key regulators having pleiotropic effects on phenotypes, as in the case of the *ERECTA* locus of *Arabidopsis* [385]. Such kind of results can contribute to answer questions about the nature of pleiotropy [386].

A strong effect of epistasis was detected in a study aimed to unravel the genetic regulation of the variation in the *Arabidopsis* metabolome. The 11 QTL clusters found in their study by Rowe *et al.* [199] influenced the accumulation of more metabolites than expected, and 8 of these clusters were associated with an epistatic network most likely regulating the plant central metabolism. The authors were also able to identify two *de novo* biochemical networks [199].

Li *et al.* [387] showed how it is already possible to generalize the genetical genomics approach. Without increasing too much the complexity of the experiments (maximum 2 factors $\times$ 2 levels, according to the authors), environmental perturbations could be introduced into a genetical genomics experiment, to understand how QTL

effects differ across multiple environments of interest (e.g., different drought locations or application of drought stress at different growth stages) and how the genotype influences the response to environmental changes.

In the same integrative view, current technical advances in high-throughput shotgun proteomics, mass spectrometry-based, should eventually allow to integrate proteomics data into phenomics ones [259]. Weckwert [388] reviewed how multivariate statistics of high-throughput shotgun proteomics and metabolomics data in *Arabidopsis*, coupled with data dimensionality reduction, can identify biomarkers (metabolites and proteins) when they result in the most important variables. Accessions could be differentiated on the basis of common and different behaviors of metabolite–protein covariances. Moreover, integrative metabolomics and proteomics studies conducted on the same sample can show that biochemical regulation is reflected by the covariance of metabolite–protein networks.

The first results obtained from long-term stress in *Arabidopsis* would suggest the importance of epigenetic regulatory mechanisms [327]. Urano *et al*. [374] reviewed the induction and repression of pathways by metabolome analysis of *Arabidopsis* cell cultures, after long-term salt stress. Relevant results obtained from the integration of omics research include the discovery of metabolic regulatory networks [374], gene regulatory networks, their coexpression modules, and key "hubs" [367], or gene coexpression networks, modules, and key "nodes" [389]. Weston *et al*. [389] emphasize the importance of substituting the two paradigms of gene-to-phenotype and phenotype-to-gene with a new one that integrates both approaches. The authors illustrate the approach of gene coexpression networks, together with concept of genomic signature. They built weighted gene coexpression networks by appropriate algorithms on the basis of abiotic stress global expression data from AtGenExpress database. They were able to obtain a list of 4000 most connected genes, with most highly connected nodes (genes) that defined 6 groups of highly correlated coexpression patterns named "modules." Owing to the general "network theory" [390], genes within coexpression modules most often share conserved biological functions. Weston *et al*. [389] identified modules responsive to heat, cold, salt, two UV-B modules, together with a coexpression group of transcripts showing significant relationships with nearly all stress treatments, overrepresented with genes participating in signal transduction, including calcium-related ones and transmembrane receptors. Interestingly, the most connected "hub" gene of such a module was a previously uncharacterized ankyrin repeat family protein, possibly regulating SA signaling. The authors conclude that relating genomic information to genetic information would be crucial for exploring the full potential of the mechanisms shaping phenotypic development [389].

Therefore, genetical genomics could contribute significantly to the dissection of the gene regulatory networks of the cell in response to abiotic stresses. The identified *trans*-acting eQTL, with caution for false positives [173], could in fact be considered as major hubs of gene regulation [173, 385]. As in the case of a unique definition for systems biology, which still does not exist, the concept of "network" also seems to acquire different appellations, representations, and meanings in the available literature. This is probably both due to the different statistical approaches pur-

sued [367] and due to the different data that originate a network – for example, Yuan *et al.* [381] describe gene-to-metabolite, "interactomics", transcriptional regulatory and gene regulatory networks. Moreover, no common rule of representation of networks was established until recently; the only common graphical rule being the presence of "nodes" and lines of connection to other nodes. That is why Le Novere *et al.* [391] recently proposed a unified Systems Biology Graphical Notation (SBGN) to provide all systems biology an acknowledged way of graphical representation.

Urano *et al.* [374] presented a new and complex regulatory network for the response of *Arabidopsis* to abiotic stresses, which might substitute the more simplistic one, going from sensor to effector genes, through signal transducers and TFs. Such a model, which includes the control by small RNAs and by the mRNA turnover, also hypothesized a role in controlling gene expression for two kinds of cytoplasmic structures, the mRNA processing body and the so-called stress granule, which contains translation initiation factors. Very recently, Street *et al.* [367] identified 38 transcriptional modules in *Populus* leaves, finding overrepresented Gene Ontology (GO) categories in 71% of them, and individuating 18 modules conserved between *Arabidopsis* and *Populus*. Interestingly, the integrative omics approach predicted new leaf transcriptional regulators; a particularly strong hub was a transcription factor putatively belonging to the Trihelix family of plant-specific transcriptional activators, predicted to be involved in the regulation of all 55 photosynthesis genes overrepresented in transcriptional modules.

One of the more ambitious goals of the systems biology is the "virtual plant project," which is aiming to generate dynamic models of a plant to describe its biological processes at different levels, from molecular to ecological [381]. Very recently, a Web-based collection of informatics tools, named "ePlant," has been released, which allows, among other features, to display three-dimensionally biological data of the model plant *Arabidopsis*, such as the three-dimensional structure of more than 70% of the *Arabidopsis* proteome [392]. Such initial accumulation of results from integrative biology thus delivers a cautious optimism about the possibilities to deepen the knowledge about the barley tolerance to abiotic stresses.

34.4.1
From Omics to Systems Biology of Abiotic Stress Tolerance in Barley

Future barley omics efforts for understanding the biology of abiotic stress responses, beyond doubt, should follow as much as possible integrative approaches, to deliver results of general meaning for the grasses and temperate cereal crops in particular. The development of the so-called "systems biology" was needed to integrate multidimensional biological information into networks and models. Therefore, after Yuan *et al.* [381] we might simply define systems biology as the study of interactions between different biological components, using models or networks. However, the future development of systems biology in crop species such as barley depends on the level of genomics knowledge and data accumulated; for this reason, there are significant expectations from the efforts of the International Barley Sequencing Consortium [16]. The simple development of a unified online analytical environ-

ment, GeneNetwork (http://www.genenetwork.org), containing barley genotypic, phenotypic, and expression profiling data represents the first attempt of barley "systems genetics" [393]. Focusing on transcriptional regulatory networks could be of particular interest for (1) the validation of predicted hubs and modules, like that of DREB1/CBF; (2) for the discovery of "hidden" regulatory hubs (see, for instance, Weston *et al.* [389]); and (3) for studying network modulation in case of multiple imposed stresses, better if in field conditions.

Another interesting perspective in our opinion is a further exciting development of quantitative genetics, within a new, integrative omics frame, in three directions. In the first one, barley genetical genomics of abiotic stress tolerance could produce significant results, mainly by deploying transcriptional profiling and metabolomics data. This because both segregating populations are designed *ad hoc*, and large characterized samples of cultivated diversity are available. The discovery of *cis*- and *trans*-acting eQTL and mQTL should help clarify the existing networks at such molecular levels. Importantly, epistatic effects, most likely existing in such complex biological responses, could be uncovered in barley/abiotic stress systems as demonstrated by Rowe *et al.* [199] in *Arabidopsis*. The proposed generalized genetical genomics approach [387] could also be pursued to dissect conditional genetic variation, that is, G × E, occurring in the barley responses to abiotic stresses. After Kliebenstein [386], some studies reported initial evidence for intriguing bias of such conditional QTL toward paralogous gene pairs. As the second direction, the hypothesis of Orr [394] could be explored. For the author it is likely that, as an alternative to the Fisher's infinitesimal theory for quantitative trait loci (not demonstrated by the accumulated QTL mapping results in various organisms), the early evolutionary steps in the random adaptive walk to the optimum would tend to be longer than those that follow. Owing to Orr's theory then, the evolutionary model leads to the robust prediction that distribution of effect sizes is exponential; quantitative variation is determined by a few QTL of (relatively) large effect and a number of genes of progressively smaller effects [395]. If the summarized theory was confirmed, on the one hand the oldest adaptive variants in barley would tend to be associated with the largest effects and, on the other hand, a large part of minor QTL effects should still remain undetected. The first significant step in this direction has been made in maize [378, 386]. Since 2008, when a Nested Association Mapping (NAM) system had been developed, by crossing 25 different founders with a common tester line, Buckler *et al.* [396] identified 333 significant loci for flowering time in a NAM population of nearly 5000 lines, and the vast majority of QTL had small to moderate effects. The third short-term perspective could be the coupling of genome-wide association studies (GWA), when more complete genomic sequence will be available, coupled with NAM or other segregating populations, like the interesting "epigenetic RILs" (epiRILs) of *Arabidopsis* [397], taking the advantages of combining association with traditional linkage mapping studies. From another point of view, it could be surveyed how much structural variants such as copy number variants (CNVs) and Indels are diffused among barley genomes, to study their impact on phenotypic quantitative variation.

The first relevant constraint in such perspective studies is the further development of barley phenomics or of precise phenotyping for abiotic stress tolerance of large

numbers of genotypes. The second constraint could derive from the apparently contrasting results that show diffused phenotyping buffering in *Arabidopsis*. Joosen *et al.* [385] report a QTL study comparing transcript, protein, and metabolite data with phenotypic traits, where only a limited number of QTL hot spots with major, system-wide effects were detected, indicating that most of the genotypic variation was phenotypically buffered. Then phenotyping, together with a further development of phenomics, should be targeted to the dissection of complex phenotypes. These could, in fact, contain "hidden," nonobvious phenotypes. Tardieu and Tuberosa [398] reviewed such issue, describing how recent studies in maize first related response curves of leaf elongation rate to temperature, evaporative demand, and soil water status, which were stable characteristics of the genotypes under different experimental conditions. The parameters of these responses were calculated for lines of mapping populations and were then genetically analyzed. On the basis of these results, they also propose this procedure as a general two-step strategy, in which phenotyping platforms could dissect complex phenotypes into simpler ones, successively being coupled to quantitative genetics and modeling studies.

To support the theory of integrative versus differential genetics, Noble [380] submits to the reader some interesting arguments, such as the concept of the DNA genomic sequence as a database of templates rather than a program, and the concept of the self-maintenance of cell structure, because it is first subjected to physics laws rather than exclusively governed by the DNA. He also reports pioneer experiments of McLaren and Michie [399], which in 1958 demonstrated how skeletal morphology (number of tail vertebrae) of different strains of mice depended on that of the mother into which the fertilized egg cell was implanted, and it could not therefore be entirely determined by the genome. Integrative views of biology and genetics then have to include genetic determinism as a component of a wider picture, which takes into account shaping of the cell and the individual in relation to the environment. Such alternative ways of regulation of stress response could be pursued in barley in the coming years. For example, the demonstrated role of (maternal) chloroplasts in cold acclimation [139] could be further studied by a systems biology approach. Taking the indication in its broadest sense, deeper studies of the noncoding RNAs, of the *cis*-regulation, of the CNVs, and of the rest of the "dispensable" genome are emphasized here as a possible perspective.

Accumulation of results on stress-induced noncoding (nc-) RNAs strongly suggest to increase also in barley the study of such level of regulation, as a fundamental element to the systems biology of environmental responses. Microarray analysis and new sequencing technologies have, in fact, revealed that the majority of the genome is transcribed in many eukaryotes. Much of the RNA appears to be noncoding and an ongoing debate is how much of a functional role it has. Matsui *et al.* [325], by *Arabidopsis* Affymetrix tiling arrays, showed that more than 7000 transcriptional units (TUs) of the "intergenic" genome were induced, of which 1275 and 181 were up- and downregulated, respectively, by the drought, cold, and high-salinity stress or ABA treatments. Most of them were shown to be hypothetical nonprotein-coding RNAs, and about 80% of the TUs were antisense transcripts fully overlapping with sense transcripts. Lu and Huang [400] reviewed how microRNAs (miRNAs),

approximately 22 nucleotide long, could be involved in abiotic stress response, and some of them strongly upregulated by cold, dehydration, and salt; interestingly, *Arabidopsis* plants constitutively expressing miR399 accumulated more inorganic phosphate than the wild-type, suggesting a role in nutrient stresses. Yao *et al.* [401] showed that some small nc-RNAs were responsive to heat, cold, salt, and dehydration in the closely related species wheat (*T. aestivum* L.). The classification and systems organization of the various noncoding RNAs is still going on. Rymarquis *et al.* [402], for example, review the class of mRNA-like noncoding RNAs (mlnc-RNAs), >40 nucleotides long, which could also be involved in response to phosphorus deprivation. De Lucia and Dean [403] recently suggested to include in this already wide scenario of noncoding small RNAs (Section 34.3.2.3) a diffused presence of long (>100 nt) noncoding RNAs, intergenic, aberrantly processed, and antisense transcripts, which can affect chromatin regulation like the small noncoding RNAs. Long nc-RNAs could influence chromatin regulation and consequent gene expression by direct effects on transcription, by recruitment of chromatin modifiers, and by formation of silent nuclear compartments. Recent data suggest that they might have interesting roles in the phenotypic plasticity of plants: the silencing by cold (vernalization) of *FLC* (*FLOWERING LOCUS C*) floral repressor gene of *Arabidopsis* seems, in fact, due to in the early phase of vernalization the COOLAIR (cold-induced long antisense intragenic RNA) antisense transcripts [403].

Plant genomes also contain conserved noncoding sequences (CNS). They are enriched in known transcription factor or other *cis*-acting binding sites. Interestingly, they are usually clustered around genes. Gene regions that respond to external stimuli are particularly rich in CNS, although only rarely does this function involve small RNA binding. It has been estimated that about 4% of *Arabidopsis* gene content is CNS-rich; such a portion of the genome, physically not irrelevant, was named as the region of "Bigfoot genes." Most Bigfoot genes in plants are transcription factors or generally "regulatory" genes. Some cases are known, such as that of the *sphingosine kinase* gene conserved in grasses (rice, sorghum, and *Brachypodium*), with CNS 12 kb upstream and 2 kb downstream chromosome [404].

As sequence information of several grass genomes started to accumulate, comparative analyses revealed that the so-called "junk DNA," largely made of transposable elements, could be responsible of a huge amount of genome variation. In fact, both intergenic and local genic content varies not only between closely related species but also among individuals within the same species [405]. One striking evidence of the *cis*-regulative role played by intergenic noncoding regions is represented by the major flowering time quantitative trait locus *Vegetative to generative transition 1* (*Vgt1*) in maize. Through positional cloning and association mapping, Salvi *et al.* [406] resolved this QTL to a mutation in a ~2 kb noncoding region positioned 70 kb upstream of ZmRap2.7, an Ap2-like transcription factor involved in flowering time control. It is today not known if such *cis*-acting regions present in noncoding intergenic regions are diffused and have a role in barley environmental responses. However, there are recent evidences of a wide diffusion of *cis*-regulation in plants, not only epigenetic, coming from genetical genomics [385], allele-specific expression [407], and *Arabidopsis* tiling array experiments [408]. As we underlined at the

beginning of this paragraph, local eQTL could be the result of closely linked *trans*-acting factors, but more often by *cis*-regulatory variation that affect transcription initiation, rate and/or transcript stability [385]. To discriminate true *cis*-regulatory eQTL from local *trans*-regulation, allele-specific expression (ASE) assays can be performed in F1 hybrid. A pioneering allele-specific expression study has been performed in the inbreeder barley by Sanger single-base extension on five F1 hybrids between putatively drought tolerant and drought susceptible parentals, including a wild *H. vulgare* ssp. *spontaneum* line. The authors used a small set of 30 genes putatively involved in stress response and verified that 63% of these showed allelic differences in expression under drought stress in at least one cross, up to a 19-fold scale, inferred to be controlled by *cis*-acting regulatory variation. The high frequency of unequal allelic expression was also influenced by genetic background, developmental stage, and drought stress [407]. These evidences would suggest the barley research community to deepen the study of the existence of and the role of diffused *cis*-regulation in tolerance to abiotic stresses. For a further unequal allelic expression study, an RNA-seq strategy should be followed once a complete array of reference sequences for each barley gene would be available for each barley chromosome.

Copy number variation is one of the reasons for differentially expressed genes in diverse individuals of a species, and this could be of phenotypic relevance. In fact, as already observed in humans, copy number variants or presence/absence variants (PAVs), possibly encompassing coding sequences, may underlie major genetic variation in traits such as susceptibility to diseases and also environmental response [409]. By short-read mapping depth, Sudmant *et al.* [410] was able to accurately estimate the absolute copy number for CNVs in 159 human genomes, reveal population diversity for CNVs, and identify human-specific expansion of genes associated with brain development. In human and *Arabidopsis*, genome-wide association scans (GWAS) used SNPs; however, known CNVs, which account for over 15% of the assembled human genome, are not easily tagged by SNPs [411]. The authors suggest the use of tailored approaches, as new-generation arrays, to explore the full genome variability beyond the SNPs. Also in plants, CNVs and PAVs might contribute to the high levels of phenotypic diversity and plasticity observed; extended surveys of structural variations are available at present for *Arabidopsis* [412] and maize [413, 414]. Through comparative genomic hybridization (CGH) array, Swanson-Wagner *et al.* [414] identified 479 genes with higher copy numbers and 3410 genes with fewer or missing copy numbers compared to the inbred line B73. In comparison with teosinte, these variants were suggested to be ancient (in agreement with Belò *et al.* [413]) and to predate domestication; many of CNV genes belonged to gene families. Belò *et al.* [413] found them distributed along all maize chromosome arms, and both groups concluded that they might have a considerable impact on maize phenotypes. By the same CGH approach, DeBolt [412] identified regions of gene CNV across the *Arabidopsis* genome. The author sought to test whether *Arabidopsis*, grown under different temperature (relatively low and high) and stress regimes (salicylic acid spray) for five generations, displayed differences in CNV relative to a plant lineage growing under normal conditions. He documented exceptional rates of CNV over immediate family generational scales, and a propensity

for duplication and nonrepetitive CNVs occurrence, which was correlated with the greatest stress conditions. This led to the hypothesized potential CNV–environment interaction, contributing to adaptive capacity of plants. Knox *et al.* [210] documented in barley CNVs in the genomic region harboring the CBF transcription factors in frost-tolerant genotypes, possibly increasing both *CBF* and downstream target-gene expression, thus resulting in a selective advantage during winter. The question if changes in *CBF* copy number are due to human selection after domestication still remains unsolved, although evidence in maize might lead to hypothesize a more general ancient origin in plants.

A single plant genome sequence might not reflect the entire genomic complement of a species as in bacteria. The concept of plant "pan-genome" was thus introduced [415], including a core and a disposable fraction. The former is composed of both single-copy sequences (represented by most if not all genes) and transposable elements that are found among all individuals in a certain genomic location, the latter is made up of partially shared and/or nonshared DNA sequence elements. In particular, the dispensable genome mostly contains transposable elements of different types that, although present in multiple copies in each individual, can be found in a specific location only in some of them. Uncovering the intriguing nature and the functional role of the dispensable genome will ultimately represent a big step toward the unraveling of the processes that are at the basis of genetic and phenotypic variation.

The list of possible perspectives could not be limited, of course, to the ones that we presented here. Importantly, the described perspective aims should also take into consideration the expected/unexpected risks and limitations present in the necessary technologies. The first risk resides in the real capacity of barley science to reach a completely postgenomics era, as already emphasized. The second big challenge is the achievement of a sufficient bioinformatic capacity, both in terms of structures and relative computational capacities and in terms of tools and researchers, which for even large barley research groups could be a significant limit. In order to cope with this challenge, more and more barley omics research groups should try to join hands with or collaborate with bioinformatics groups. Notwithstanding the limitations, trying to scale up the knowledge of the tolerance mechanisms from loci/genes/metabolites to systems is one of the most interesting promises of systems biology of stresses. As summarized by Hirayama and Shinozaki [327], among the next challenges that systems biology will face there could be the identification of abiotic stress sensors, understanding the molecular basis of interplay among stresses (including biotic ones), identification of key factors connecting abiotic stress responses and developmental processes, studying long-term plant responses under multiple abiotic stress conditions in nature.

However, even if such promises from the integrative approaches will be met, at least in the model plants, still new tools and strategies are needed to improve barley for closing the yield gap, from actual to potential yields, in the presence of abiotic stresses. We present in Figure 34.2 an updated schema of the integration of "omics" in barley, to approach the improvement of abiotic stress tolerance through an integrative biology perspective. In particular, connections between omics

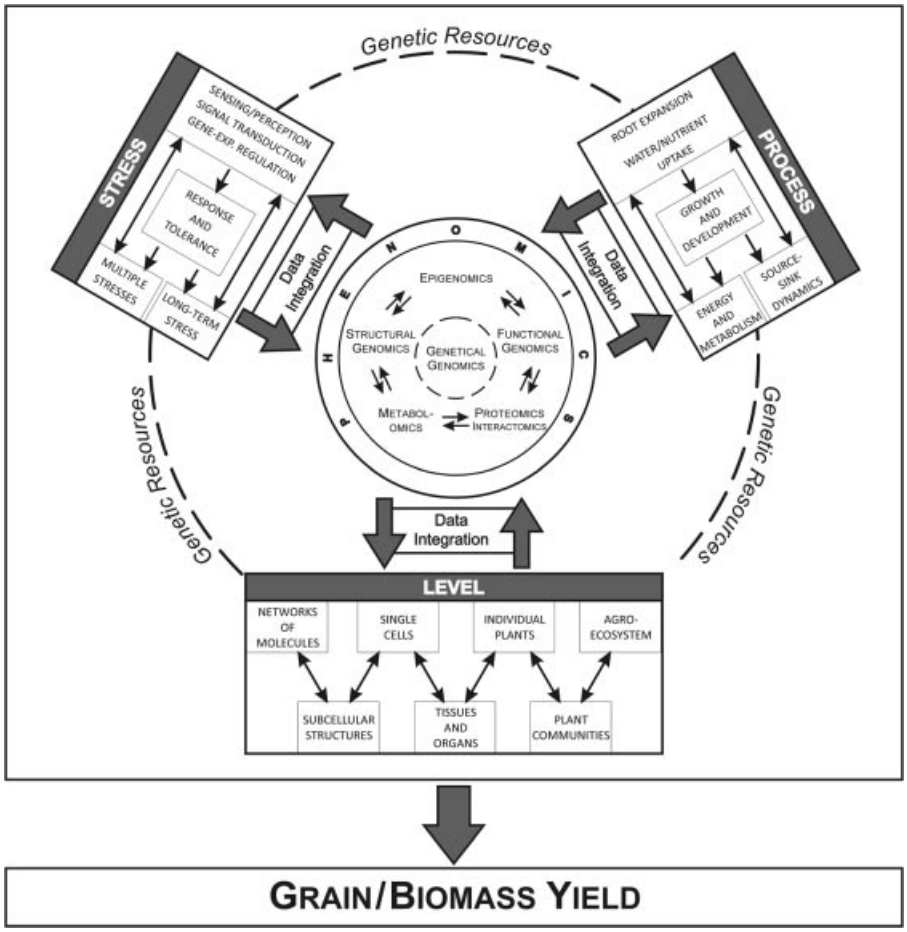

Figure 34.2 Proposed scheme of integration of "omics" sciences within a frame of systems biology, for the improvement of barley abiotic stress tolerance.

components, crop physiology, and barley genomics-assisted improvement have been introduced into the schema.

34.4.2 Integration of Omics with Crop Science

As for other crops, the ultimate objective for barley research is yield or, more precisely, potential grain/biomass yield. This is the first unquestionable difference from plant models like *Arabidopsis* or *Brachypodium*. The second difference lies in barley's relationship with the environment, through all its growth cycle till its final potential yield, and the interaction with the agricultural soil; hydroponics or other artificial growth conditions are not an option. It is worth noting that for the

identification of the genetic basis of adaptive variation, *Arabidopsis* research is still aiming as a perspective to reach ecologically realistic conditions, by simulating outdoor field experiments [327, 397]. For example, through a genome-wide association study, Bergelson and Roux [397] found that only 2 out of 25 candidate genes associated with flowering in *Arabidopsis* and measured under field conditions had also been proposed as candidate genes for flowering time scored under greenhouse conditions. The third difference is that barley, as a cultivated plant, went through a long history of domestication and breeding practically in all regions across the world, which led to a large number of genomic rearrangements named cultivars (see also Section 34.2) that are not available for the noncrop model plants. The last and maybe most striking difference, from a plant biology point of view, is that all barley relations with the environment should be considered as the interactions of a community of barley plants with the environment and between each other with respect to the environment. Although this is true in a certain aspect also for *Arabidopsis* and *Brachypodium* in nature, where they live in mixed interspecific communities, it is not the case of their experimental systems and studies, where the individual level has been practically always the highest level of investigation. Of course, all these differences cannot be taken in account for rice, the third genomic model of reference for cereals that in the four mentioned aspects can be considered identical to barley.

As a consequence of such differences, the phenotypic complexity compared to a noncrop model is significantly higher. Phenotypes at the crop (community) level are expected to be regulated by gene networks whose effects and expression could be highly dependent on environmental conditions and developmental stages. These phenotypes are achieved through multiple intermediate component processes and orchestrated feedback mechanisms, by both intra- and interplant competition and by interactions between stress factors. Because of the competition, a change of one component may result in an often unexpected, but negative, consequence on other components, and the final yield of a crop cannot be simply predicted from the yield of single plants grown in isolation [416]. For these reasons, the study of the dynamics of response to abiotic stresses in long-term experiments, in different developmental phases, and in field conditions of competition with other individuals, although representing a highly complex experimental scenario, could be crucial to obtain affordable data on regulation networks. This, in turn, would be useful to depict the reality of the crop. The importance of scaling up the systems biology from molecules to ecosystems has been emphasized recently. Raes and Bork [383], even if presenting a microbial ecosystems biology (easier to be dealt with), correctly underscore how any systems biology approach would need a "parts list," the connectivity between the parts, and the placement of such connectivity in a context of time and space. Keurentjes *et al.* [384], while presenting fascinating dynamic response scenarios in plants, where volatile organic compounds and insects are involved, invoke the challenge to generate accurate experimental data that can be used together with modeling concepts in a modeling–validation cycle. Very recently, Li *et al.* [417] studied the developmental dynamics of the maize leaf transcriptome using Illumina sequencing platform. They quantified transcript abundance along a leaf developmental gradient, detecting differential mRNA processing events for most maize

genes. The authors found that 64 and 21% of genes were differentially expressed along the developmental gradient and between bundle sheath and mesophyll cells, respectively. The data set they created could serve as the foundation for a dynamic systems biology approach for understanding photosynthetic development.

While ecosystems biology is still at its first steps, the discipline of crop physiology can be considered fully developed and available at present. Crop physiology, also defined as the physiology of the crop plant communities, was in fact directly supported by the described peculiarities of the crop systems. Crop physiologists have substantially developed models predicting the behavior of a crop at varying doses of external inputs, such as fertilizers, solar radiation, or water. At its beginning, de Wit introduced in the late 1960s–early 1970s into crop physiology the "general systems theory" of von Bertalanffy, first developed in the 1930s, and simulation methodologies of Forrester, acquired in the 1960s and initiated research for modeling the whole crop. The development of the whole-crop physiology modeling in the late 1960s was then analogous to the initiative for developing systems biology today, as regard the need for instruments that could summarize increasing quantities of experimental data.

A notable example of success of crop modeling to influence breeding is the development of the so-called "super rice" in China. Setter *et al.* [418] showed, thanks to a crop model, that thick erect final leaves displayed well above a low panicle were essential for high yields in rice. This ideotype concept, combined with the use of heterosis and of wider genetic resources, led in China to the release to farmers of several super rice varieties, both inbred and F1 hybrid, some of them widely grown in China [416]. Crop physiology design could help bring further success in rice yields. A few years ago, it was suggested that supercharging photosynthesis could be the only way to improve substantially yield potential in rice while not increasing the demand for water and nitrogen [419]. These authors strongly proposed introducing into rice the C4 biochemical pathway and modifying leaf anatomy so that the C4 system works at its best in this crop. To examine the hypothesis, a detailed crop model analysis was performed to assess the impact of the full C4 system introduced into C3 rice. The simulations, conducted under the conditions of the International Rice Research Institute (IRRI) experimental farm, envisaged a yield advantage of C4 versus C3 rice from 10.5 ton ha^{-1}, the maximum rice yield observed at the IRRI farm, to 13.1 ton ha^{-1}. This would represent an increase of 23%, a significant although lower than the 50% increase hoped for by Mitchell and Sheehy [419]. In addition, in contrast to these authors' proposition, owing to modeling conclusions, a higher nitrogen uptake would be needed to reach a higher yield because C4 photosynthesis responds more to leaf nitrogen than to the C3 routine under high light conditions.

Systems biology of plants should then try to add the last layer (crop community) of the hierarchical organization of living beings; this is necessary in view of the needed future contribution to food production and energy supply improvement. In the present situation, crop physiology showed its possibilities, although it did not make use at least until now, of information from lower hierarchical levels. There could be therefore, also for barley science, a need to follow the new route of "crop systems biology" [416]. The concept was proposed aiming at modeling complex crop-level

traits relevant to yield potential, by building links between "omics"-level information, underlying biochemical understanding, and physiological component processes. Developing crop systems biology could be demanding and with many challenges. Mapping the organization levels (parts) and the communication systems between these levels for the different key processes could become too complicated. However, a robust model may not be necessarily a complex one and much of the fine details may not be needed [420].

In the view of reducing complexity, the integration of genetic and genomics data into crop physiology models could represent a possibility. We are convinced that time is ripe for such an approach in barley, where a wealth of data are available on both sides, that of field adaptation to multiple stresses and that of genomics information. It could be simply needed, for instance, to refine crop physiology models introducing the genomic constitution as a further module of variation. On the basis of the qualitative genetic characterization of major flowering time genes in *Arabidopsis*, Welch *et al*. [421] proposed preliminary quantitative neural network model of flowering time control in this species. Similar modeling might be explored for phenology in barley both in temperate and in severely physically stressed environments. It is worth noting that scaling down to a lower level is not a one-directional process that "omicists" alone could do, without the modeling experiences of crop physiologists. For more complex candidate traits/processes than phenology, such as carbon assimilation, nitrogen assimilation, and stress tolerance response itself, a fine understanding of systems biology first could be more urgent.

34.4.3
Integration of Omics with Future Barley Breeding for Abiotic Stress Tolerance

In the opening pages of this chapter, we hoped that the knowledge of the barley tolerance to abiotic stresses obtained thanks to the omics research could have been used to implement MAS programs of new varieties. Engineering barley to abiotic stress tolerance could be a viable option, as well as for other plants, quite recently reviewed with its achievements and limitations by Vinocur and Altman [90]; however, this would be expected mainly for those countries with a fair law regulatory system. If we define MAS as the genotypic selection of individuals performed in addition and eventually substitution of phenotypic selection, it is obvious that we need detectable DNA variations in linkage to genes and QTL responsible for agronomically relevant traits. In this way, it would be possible to select DNA sequence variants (i.e., alleles of genetic markers) without the need to phenotype the agronomical effect of the gene/QTL (for a review, see Ref. [422]). Some concepts and observations can be derived from such a definition. First, this approach for barley improvement is based on the differential genetics theory [380]. On the basis of the gene-centric differential theory, in fact, different molecular, and eventually agronomical, field-scored phenotypes should correspond to different DNA variants at each gene. Such deterministic and simplistic vision of genetics is not anymore sufficient to describe the reality of complex phenotypes, as it was previously said. Consequently, MAS technology as it has been considered so far could not be anymore considered as totally valid. The

second observation is about which loci of barley should be selected. Figure 34.1 partly answers the question. Cloned genes, candidate genes to explain components of tolerance, described in the previous sections, are the first sequences whose positive variants could be selected. In addition to such key determinants with generally qualitative or major effects on tolerance, the genomic regions harboring positive alleles of QTL for abiotic stress tolerance could be introgressed, one by one, or "pyramided" in higher numbers into selected genotypes. In this case, classically two flanking markers for each QTL should be used since every QTL is mapped at one most significant locus, but with a confidence interval around the most likely position. Efficient MAS, verified and validated in the past years by a large number of plant breeders through the field performances of the marker-selected genotypes, is a demonstration that in this case the deterministic differential concept still holds, without the apparent need of an integrative approach to the matter. Examples of barley MAS based on a few key loci are very numerous, from tolerance to several biotic stresses (see, for example, Ref. [423]), to malting quality traits (see, for example, Ref. [424]), grain yield (see, for example, Ref. [425]), and tolerance to abiotic stresses (see, for example, Ref. [426–428]). Briefly, not every DNA polymorphism in a candidate gene is able to discriminate tolerant genotypes; every sequence variation, or, better, every SNP haplotype recognized in a gene sequence must be first validated as associated with superior phenotypes in a panel of donor and recipient breeding lines and cultivars, before considering it as an MAS tool. For example, while it was demonstrated that the *CBF* gene cluster at *FR-H2* was conferring frost tolerance in a "winter" × "spring" type barley cross (see Section 34.2.5.2), when the *CBF* polymorphisms were used for MAS in a panel of facultative, winter, and spring barleys [427], or in a panel of winter barleys [428], only few of them were found slightly associated with frost-tolerant genotypes. On the contrary, the mapping polymorphism of the current candidate gene for *Fr-H1* frost tolerance QTL, the vernalization gene *HvBM5A*(*VRN-H1*) [214] was indeed highly associated with tolerant phenotypes on the same panel of barleys, and could be used for MAS [427].

The effect of the selected allele in these cases is simply mechanistic; once inherited in a plant, it simply determines an improvement for the trait of such an individual. As underlined by Weston *et al.* [389], one of the major opportunities afforded by modern genomics technologies, which ecology was not able to afford, is in fact the potential to provide a mechanistic understanding of the processes by which genetic change translates into phenotypic variation and the resultant appearance of distinct physiological traits. For example, in medicine genomic information can be used to determine the physiological state (e.g., diagnosis) and predict phenotypic outcome (e.g., patient survival). It is then worth to be concluded that once composed in their positive allelic variants into a plant, a relatively small number of candidate genes playing major effects on stress tolerance, could be sufficient to significantly improve existing crop varieties. Notably in rice, the simple introgression by marker-assisted backcrossing of the cloned *Sub1* QTL of submergence tolerance successfully improved such a trait in a rice cultivar widely diffused in flood-prone Asiatic regions [429]. Recently, Francia *et al.* [225] demonstrated how a simple allelic combination of three candidate genes for the induction of flowering (that reside

at *VRN-H1*, *EAM6*, and *PPD-H2*) could be selected while keeping randomly unselected the rest of the genome. Through the regulation of the duration of the growth cycle phases, such an allelic combination was *per se* sufficient to gain $\sim$0.2 ton ha^{-1} compared to the average grain yield recorded for the "Nure" $\times$ "Tremois" population in 18 Mediterranean locations, from fertile to heavy drought environments. When such a few-gene combination would be inserted into low-cost MAS programs, it might constitute a precision stress "escape" breeding strategy, until now not pursued. The Generation Challenge Program nicely followed a similar aim, by producing a barley marker-toolbox accessible by laboratories of developing countries, which contains simple PCR-based markers for the introgression of key genes improving fundamental agronomic traits (The Generation Challenge Programme's Molecular Marker Toolkit is curated by V. van Damme, [430]). However, it might be investigated up to which number of such key loci should be cumulated into a genotype until either a plateau or a full potential yield is reached. The two issues were not investigated in detail in barley, at least with the specific aim of their theoretical validation.

Since some years, scaling up MAS has been suggested in crops till the genome level, namely, to perform genomics-assisted selection [431]. In principle, at least one polymorphism, or, to be more informative, one haplotype per barley gene could be used, reaching tens to hundreds of thousands scorable SNPs. Intermediate levels could also exist, in which, for example, all the abiotic stress-induced or related genes could be selected, rather than all the markers in the genome.

The efficiency of improving barley by genomics-assisted selection has still not been demonstrated. We could not consider in this chapter the big challenge to obtain through recombination in this homozygously cultivated selfer species a precise combination, in *coupling*, of positive alleles, at all loci. Then, at least theoretically, such a "designed" genome [432] could be obtained. Another heavy task before starting genomics-assisted selection is not absolutely trivial; the necessary step is to associate with a trait each locus that should be selected, and to identify the sequence variant (allele) with a putative positive effect on the trait. Once one would be able to obtain the desired allelic combination at thousands of loci, it could be verified which is the overall performance of the selected genotype; conversely, if there are or not interaction effects between the selected genes and the genetic background. In fact, it is possible that once constituted in its final shape, such a genomic ideotype not only would keep the promise to increase performances but also it could lose some and gain new interaction effects, which together would not allow it to reach the desired performance.

QTL MAS deserves a separate discussion. If the Orr's exponential theory is correct, the majority of the QTL that were mapped until now in barley could be major-to-moderate effect QTL, and still a significant part of the quantitative variation would be missing, contributed by a large number of minor-effect QTL. On the one hand, this would not exclude, at least for the moment, the Robertson's hypothesis about the Mendelian nature of genes underlying QTL, as a sort of "defeated" alleles of qualitative effect genes. As underlined by Collins *et al.* [378], the majority of the QTL cloned in barley and in other crops until today are in agreement with such a

theory. In this view, the major and moderate effect QTL could be the regulatory hubs identified by functional genomics in the stress response pathways, and by genetical genomics. On the other hand, the identification of a large number of minor QTL could be challenging, since crossing schemes such as NAM, coupled with a huge phenotyping work on thousands of genotypes, could be required. Another hypothesis should also be made on the nature of the minor QTL. If it could be verified that major-to-moderate effect loci are in general agreement with Robertson's theory and they are variants of Mendelian single genes, we might conclude that they reside in the so-called "core" genome [415], and this could be conceivable for all QTL that were acquired through the barley domestication history. It might be argued if CNV could be important for explaining the biological bases of QTL, as hypothesized in *Arabidopsis* [397, 412], and in one case in barley [210], and which of them and in which part of the genome could be relevant for adaptation to stresses. Then, it might be argued if minor QTL, once accurately mapped, would reside in the "dispensable" genome rather than in the core one. In other words, it could be verified if their nature is genic, structural-based, or based on noncoding regulatory regions.

The results of barley genome sequencing [16] could help verify the existence and characteristics of the dispensable genome also in this autogamous species and to compare its features with what has been observed in an allogamous species such as maize [415]; consequently, the hypothesis about the "real" nature of minor QTL could also be verified. To accomplish this task, genomics sequencing would need to be coupled with GWA and NAM studies on large populations, and with phenomics of tolerance to abiotic stresses [378, 397, 398].

We have already shown in these last sections how a redirection of the traditional "reductionist science" approach to a "systems biology" approach is required to face agricultural challenges in the future. Indeed, the bulk of the potential for future improvements in barley tolerance to abiotic stress lies in the elucidation and understanding of interactions of the various components of the biology of the plant in concert with all the parameters of the production environment. First of all, systems biology could tell us more about interactions about loci, and move molecular-assisted breeding from the differential view of genetics to a comprehensive integrative approach. Second, it might help take barley from actual to potential yields, a fact that could become more urgent in coming years. In fact, while it was demonstrated that a combination of few loci would be sufficient to reach elite material yields by MAS, as already said, genomics-assisted selection of an *a priori* fixed genomic allele combination (ideotype) might be not effective to reach potential yields. Interactions between coding loci, between these and noncoding loci, and between these and other factors, for instance, cellular structures, could play a significant role to reach the final phenotype. For instance, Joosen *et al.* [385] report a metabolome study where more than one-third of the compounds present in the RILs were not detected in either parent but were the most likely result of recombination of loci contributing to biosynthesis pathways. Systems biology, by integrating the information at the various molecular and structural levels, could also contribute to a better understanding and add components of stress avoidance [90] strategies to the genomics-assisted selection.

The last issue should be discussed: the potential need for new MAS tools at reasonable costs. As in other major crops, barley MAS is used both in the public and in the private sector, in Europe and in other countries, mainly for introgressing disease resistances and quality traits [422]. At present, different MAS platforms are available, based on different technologies, although in many cases they are not really cost-effective. In the case of SNP array platforms, they not always have sufficient flexibility for user-defined SNPs that might vary between different projects. If we focus on the barley and wheat seed market, we might realize that it is a market of self-pollinating crops in which conditions of agricultural economical crisis could lower the percentage of certified seed to less than 50% [433] and in which not all the seed companies would invest huge amounts of money due to the possible low returns of the investments. This notwithstanding, we believe that it is mandatory both to increase the precision of breeding by genomics MAS and to include abiotic stress tolerance into breeding targets. Moreover, there could be a place for the public research to produce high-throughput although flexible genotyping platforms, which at the same time could contain as much as possible the costs of genotyping.

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References

1 Levine, S. (2005) Developmental determinants of sensitivity and resistance to stress. *Psychoneuroendocrinology*, **30**, 939–946.
2 Larcher, W. (1987) Stress bei Pflanzen. *Naturwissenschaften*, **74**, 158–167.
3 Pahlich, E. (1993) Larcher's definition of plant stress: a valuable principle for metabolic adaptability research. *Braz. J. Plant Physiol.*, **5** (2), 209–216.
4 Abeledo, L.G., Calderoni, D.F., and Slafer, G.A. (2003) Genetic improvement of barley yield potential and its physiological determinants in Argentina (1944–1998). *Euphytica*, **130** (3), 325–334.
5 Stanca, A.M., Romagosa, I., Takeda, K., Lundborg, T., Terzi, V., and Cattivelli, L. (2003) Diversity in abiotic stress tolerances, in *Developments in Plant Genetics and Breeding: Diversity in Barley Hordeum vulgare* (eds R. von Bothmer, T. van Hintum, H. Knupffer, and K. Sato), Elsevier Science BV, Amsterdam, pp. 179–199.
6 Pecchioni, N., Cattivelli, L., Delogu, G., Faccioli, P., Terzi, V., Vale, G., and Stanca, A.M. (2002) Barley, in *Evolution and Adaptation of Cereal Crops* (eds V.L. Chopra and S. Prakash), Science Publishers Inc., Enfield, pp. 135–211.
7 Levitt, J. (1980) *Responses of Plants to Environmental Stresses*, Academic Press, New York.
8 Reinheimer, J.L., Barr, A.R., and Eglinton, J.K. (2004) QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, **109**, 1267–1274.
9 Jefferies, S.P., Barr, A.R., Karakousis, A., Kretschmer, J.M., Manning, S., Chalmers, K.J., Nelson, J.C.,

Islam, A.K.M.R., and Langridge, P. (1999) Mapping of chromosomal regions conferring boron toxicity tolerance in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, **98**, 1293–1303.

10 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.*, **17** (3), 229–230.

11 Xu, Z.S., Xia, L.Q., Chen, M., Cheng, X.G., Zhang, R.Y., Li, L.C., Zhao, Y.X., Lu, Y., Ni, Z.Y., Liu, L., Qiu, Z.G., and Ma, Y.Z. (2007) Isolation and molecular characterization of the *Triticum aestivum* L. ethylene-responsive factor 1 (TaERF1) that increases multiple stress tolerance. *Plant Mol. Biol.*, **65** (6), 719–732.

12 Mittler, R. (2006) Abiotic stress: the field environment and stress combination. *Trends Plant Sci.*, **11** (1), 15–19.

13 Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., and Mittler, R. (2004) When defense pathways collide: the response of *Arabidopsis* to a combination of drought and heat stress. *Plant Physiol.*, **134**, 1–14.

14 Walia, H., Wilson, C., Condamine, P., Liu, X., Ismail, A.M., and Close, T.J. (2007) Large-scale expression profiling and physiological characterization of jasmonic acid-mediated adaptation of barley to salinity stress. *Plant Cell Environ.*, **30**, 410–421.

15 Stein, N. (2007) Triticeae genomics: advances in sequence analysis of large genome cereal crops. *Chromosome Res.*, **15**, 21–31.

16 Schulte, D., Close, T.J., Graner, A., Langridge, P., Matsumoto, T., Muehlbauer, G., Sato, K., Schulman, A.H., Waugh, R., Wise, R.P., and Stein, N. (2009) The International Barley Sequencing Consortium: at the threshold of efficient access to the barley genome. *Plant Physiol.*, **149**, 142–147.

17 Langridge, P., Paltridge, N., and Fincher, G. (2006) Functional genomics of abiotic stress tolerance in cereals. *Brief. Funct. Genomic Proteomic*, **4** (4), 343–354.

18 Sreenivasulu, N., Sopory, S.K., and Kavi Kishor, P.B. (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. *Gene*, **388**, 1–13.

19 Jensen, D.B., Torn, M., and Harte, J. (1990) In our own hands: a strategy for conserving biological diversity in California, California Policy Seminar Research Report, University of California, Berkeley.

20 von Bothmer, R., Sato, K., Komatsuda, T., Yasuda, S., and Fischbeck, G. (2003) The domestication of cultivated barley, in *Developments in Plant Genetics and Breeding: Diversity in Barley Hordeum vulgare* (eds R. von Bothmer, T. van Hintum, H. Knupffer, and K. Sato), Elsevier Science BV, Amsterdam, pp. 3–280.

21 Schloss, P.D. and Handelsman, J. (2003) Biotechnological prospects from metagenomics. *Curr. Opin. Biotechnol.*, **14** (3), 303–310.

22 Shendure, J. and Ji, H. (2008) Next-generation DNA sequencing. *Nat. Biotechnol.*, **26**, 1135–1145.

23 Metzker, M. (2010) Sequencing technologies. *Nat. Rev.*, **11**, 31–46.

24 Tanksley, S.D. and McCouch, S.R. (1997) Seed banks and molecular maps: unlocking genetic potential from the wild. *Science*, **277** (5329), 1063–1066.

25 Knupffer, H., Terentyeva, I., Hammer, K., Kovaleva, O., and Sato, K. (2003) Ecogeographical diversity: a Vavilovian approach, in *Development in Plant Genetics and Breeding: Diversity in Barley Hordeum vulgare* (eds R. von Bothmer, T. van Hintum, H. Knupffer, and K. Sato), Elsevier Science BV, Amsterdam, pp. 53–76.

26 Global Crop Diversity Trust (2008) Global strategy for the *ex situ* conservation and use of barley germplasm http://www.croptrust.org/documents/web/Barley_Strategy_FINAL_27Oct08.pdf (8 February 2011).

27 http://www.nordgen.org/index.php/en/content/view/full/371 (8 February 2011).

28 Costa, J.M., Corey, A., Hayes, P.M., Jobet, C., Kleinhofs, A., Kopisch, A., Kramer, S.F., Kudrna, D., Li, M., Riera-Lizarazu, O., Sato, K., Szucs, P., Toojinda, T., Vales,

M.I., and Wolfe, R.I. (2001) Molecular mapping of the Oregon Wolfe Barleys: a phenotypically polymorphic doubled-haploid population. *Theor. Appl. Genet.*, **103**, 415–424.

29 http://earth.lab.nig.ac.jp/~dclust/cgi-bin/BCC (8 February 2011).

30 Knupffer, H. and van Hintum, T. (2003) Summarised diversity: the barley core collection, in *Developments in Plant Genetics and Breeding: Diversity in Barley Hordeum vulgare* (eds R. von Bothmer, T. van Hintum, H. Knupffer, and K. Sato), Elsevier Science BV, Amsterdam, pp. 259–267.

31 Nevo, E. (1992) Origin, evolution, population genetics and resources for breeding of wild barley, *Hordeum spontaneum*, in the Fertile Crescent, in *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology* (ed. P.R. Shewry), CAB International, The Alden Press, Oxford, pp. 19–43.

32 Shavrukov, Y., Gupta, N.K., Miyazaki, J., Baho, M.N., Chalmers, K.J., Tester, M., Langridge, P., and Collins, N.C. (2010) *HvNax3*-a locus controlling shoot sodium exclusion derived from wild barley (*Hordeum vulgare* ssp. *spontaneum*). *Funct. Integr. Genomics*, **10** (2), 277–291.

33 Kumar, G.R., Sakthivel, K., Sundaram, R.M., Neeraja, C.N., Balachandran, S.M., Rani, N.S., Viraktamath, B.C., and Madhav, M.S. (2010) Allele mining in crops: prospects and potentials. *Biotechnol. Adv.*, **28** (4), 451–461.

34 Kaur, K., Street, K., Mackay, M., Yahiaoui, N., and Keller, B. (2008) Allele mining and sequence diversity at the wheat powdery mildew resistance locus Pm3, in *Proceedings of the 11th International Wheat Genetics Symposium* (eds R. Appels, R. Eastwood, E. Lagudah, O. Langridge, M. Mackay, and L. McIntyre), Sydney University Press, Sydney, pp. 1–3.

35 Cockram, J., Chiapparino, E., Taylor, S.A., Stamati, K., Donini, P., Laurie, D.A., and O'Sullivan, D.M. (2007) Haplotype analysis of vernalization loci in European barley germplasm reveals novel VRN-H1 alleles and a predominant winter VRN-H1/VRN-H2 multi-locus haplotypes. *Theor. Appl. Genet.*, **115**, 993–1001.

36 Upadhyaya, H.D. and Ortiz, R. (2001) A mini core subset for capturing diversity and promoting utilization of chickpea genetic resources in crop improvement. *Theor. Appl. Genet.*, **102** (8), 1292–1298.

37 Park, Y.J. (2007) Allele mining experience on rice germplasm through advanced M strategy using modified heuristic search. W9, Plant and Animal Genomes XV Conference, January 13–17, 2007, San Diego.

38 Bennett, M.D. and Smith, J.B. (1976) Nuclear DNA amounts in angiosperms. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **274**, 227–274.

39 Wicker, T., Schlagenhauf, E., Graner, A., Close, T.J., Keller, B., and Stein, N. (2006) 454 sequencing put to the test using the complex genome of barley. *BMC Genomics*, **7**, 275.

40 Wicker, T., Narechania, A., Sabot, F., Stein, J., Vu, G.T.H., Graner, A., Ware, D., and Stein, N. (2008) Low-pass shotgun sequencing of the barley genome facilitates rapid identification of genes, conserved non-coding sequences and novel repeats. *BMC Genomics*, **9**, 518.

41 Steuernagel, B., Taudien, S., Gundlach, H., Seidel, M., Ariyadasa, R., Schulte, D., Petzold, A., Felder, M., Graner, A., Scholz, U., Mayer, K.F.X., Platzer, M., and Stein, N. (2009) *De novo* 454 sequencing of barcoded BAC pools for comprehensive gene survey and genome analysis in the complex genome of barley. *BMC Genomics*, **10**, 547.

42 Wicker, T., Taudien, S., Houben, A., Keller, B., Graner, A., Platzer, M., and Stein, N. (2009) A whole-genome snapshot of 454 sequences exposes the composition of the barley genome and provides evidence for parallel evolution of genome size in wheat and barley. *Plant J.*, **59**, 712–722.

43 Heun, M., Kennedy, A.E., Anderson, J.A., Lapitan, N.L.V., Sorrells, M.E., and Tanksley, S.D. (1991) Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). *Genome*, **34** (3), 437–447.

44 Rostoks, N., Mudie, S., Cardle, L., Russell, J., Ramsay, L., Booth, A., Svensson, J.T., Wanamaker, S.I., Walia, H., Rodriguez, E.M., Hedley, P.E., Liu, H., Morris, J., Close, T.J., Marshall, D.F., and Waugh, R. (2005) Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress. *Mol. Genet. Genomics*, **274**, 515–527.

45 Marcel, T.C., Varshney, R.K., Barbieri, M., Jafary, H., de Kock, M.J.D., Graner, A., and Niks, R.E. (2007) A high-density consensus map of barley to compare the distribution of QTLs for partial resistance of *Puccinia hordei* and of defence gene homologues. *Theor. Appl. Genet.*, **114**, 487–500.

46 Varshney, R.K., Marcel, T.C., Ramsay, L., Russell, J., Roder, M.S., Stein, N., Waugh, R., Langridge, P., Niks, R.E., and Graner, A. (2007) A high density barley microsatellite consensus map with 775 SSR loci. *Theor. Appl. Genet.*, **114**, 1091–1103.

47 Wenzl, P., Carling, J., Kudrna, D., Jaccoud, D., Huttner, E., Kleinhohs, A., and Kilian, A. (2004) Diversity Array Technology (DArT) for whole-genome profiling of barley. *Proc. Natl. Acad. Sci. USA*, **101** (26), 9915–9920.

48 Wenzl, P., Li, H., Carling, J., Zhou, M., Raman, H., Paul, E., Hearnden, P., Maier, C., Xia, L., Caig, V., Ovesna, J., Cakir, M., Poulsen, D., Wang, J., Raman, R., Smith, K.P., Muehlbauer, G.J., Chalmers, K.J., Kleinhofs, A., Huttner, E., and Kilian, A. (2006) A high-density consensus map of barley linking DArT markers to SSR, RFLP and STS loci and agricultural traits. *BMC Genomics*, **7** 206.

49 Hearnden, P.R., Eckelmann, P.J., McMichael, G.L., Hayden, M.J., Eglinton, J.K., and Chalmers, K.J. (2007) A genetic map of 1,000 SSR and DArT markers in a wide barley cross. *Theor. Appl. Genet.*, **115**, 383–391.

50 Potokina, E., Druka, A., Luo, Z., Wise, R., Waugh, R., and Kearsey, M. (2008) Gene expression quantitative trait locus analysis of 16,000 barley genes reveals a complex pattern of genome-wide transcriptional regulation. *Plant J.*, **53**, 90–101.

51 Pecchioni, N. (1999) EST development in barley from a cold-induced cDNA library. 9th ITMI International Triticeae Mapping Initiative Meeting, August 24–28, Viterbo.

52 Zhang, H., Sreenivasulu, N., Weschke, W., Stein, N., Rudd, S., Radchuk, V., Potokina, E., Scholz, U., Schweizer, P., Zierod, U., Langridge, P., Varshney, R.K., Wobus, U., and Graner, A. (2004) Large-scale analysis of the barley transcriptome based on expressed sequence tags. *Plant J.*, **40**, 276–290.

53 Druka, A., Muehlbauer, G., Druka, I., Caldo, R., Baumann, U., Rostoks, N., Schreiber, A., Wise, R., Close, T., Kleinhofs, A., Graner, A., Schulman, A., Langridge, P., Sato, K., Hayes, P., McNicol, J., Marshall, D., and Waugh, R. (2006) An atlas of gene expression from seed to seed through barley development. *Funct. Integr. Genomics*, **6** 202–211.

54 Tondelli, A., Francia, E., Barabaschi, D., Aprile, A., Skinner, J.S., Stockinger, E.J., Stanca, A.M., and Pecchioni, N. (2006) Mapping regulatory genes as candidates for cold and drought stress tolerance in barley. *Theor. Appl. Genet.*, **112**, 445–454.

55 Stein, N., Prasad, M., Scholz, U., Thiel, T., Zhang, H., Wolf, M., Kota, R., Varshney, R.K., Perovic, D., and Graner, A. (2007) A 1000 loci transcript map of the barley genome: new anchoring points for integrative grass genomics. *Theor. Appl. Genet.*, **114**, 823–839.

56 Sato, K., Nankaku, N., and Takeda, K. (2009) A high-density transcript linkage map of barley derived from a single population. *Heredity*, **103**, 110–117.

57 Close, T.J., Bhat, P.R., Lonardi, S., Wu, Y., Rostoks, N., Ramsay, L., Druka, A., Stein, N., Svensson, J.T., Wanamaker, S., Bozdag, S., Roose, M.L., Moscou, M.J., Chao, S., Varshney, R.K., Szucs, P., Sato, K., Hayes, P.M., Matthews, D.E., Kleinhofs, A., Muehlbauer, G.J., DeYoung, J., Marshall, D.F., Madishetty, K., Fenton, R.D., Condamine, P., Graner, A., and Waugh, R. (2009) Development and implementation of high-throughput

SNP genotyping in barley. *BMC Genomics*, **10** 582.

58 Huang, X., Feng, Q., Qian, Q., Zhao, Q., Wang, L., Wang, A., Guan, J., Fan, D., Weng, Q., Huang, T., Dong, G., Sang, T., and Han, B. (2009) High-throughput genotyping by whole-genome resequencing. *Genome Res.*, **19**, 1068–1076.

59 Sreenivasulu, N., Graner, A., and Wobus, U. (2008) Barley genomics: an overview. *Int. J. Plant Genomics*. doi: 10.1155/2008/486258

60 Yu, Y., Tomkins, J.P., Waugh, R., Frisch, D.A., Kudrna, D., Kleinhofs, A., Brueggeman, R.S., Muehlbauer, G.J., Wise, R.P., and Wing, R.A. (2000) A bacterial artificial chromosome library for barley (*Hordeum vulgare* L.) and the identification of clones containing putative resistance genes. *Theor. Appl. Genet.*, **101**, 1093–1099.

61 Isidore, E., Scherrer, B., Bellec, A., Budin, K., Faivre-Rampant, P., Waugh, R., Keller, B., Caboche, M., Feuillet, C., and Chalhoub, B. (2005) Direct targeting and rapid isolation of BAC clones spanning a defined chromosome region. *Funct. Integr. Genomics*, **5**, 97–103.

62 Saisho, D., Myoraku, E., Kawasaki, S., Sato, K., and Takeda, K. (2007) Construction and characterization of a bacterial artificial chromosome (BAC) library from the Japanese malting barley variety "Haruna Nijo". *Breeding Sci.*, **57**, 29–38.

63 Shi, B.J., Sutton, T., Collins, N.C., Pallotta, M., and Langridge, P. (2010) Construction of a barley bacterial artificial chromosome library suitable for cloning genes for boron tolerance, sodium exclusion and high grain zinc content. *Plant Breed.*, **129**, 291–296.

64 Cho, S., Garvin, D.F., and Muehlbauer, G.J. (2006) Transcriptome analysis and physical mapping of barley genes in wheat: barley chromosome addition lines. *Genetics*, **172**, 1277–1285.

65 Varshney, R.K., Grosse, I., Hahnel, U., Siefken, R., Prasad, M., Stein, N., Langridge, P., Altschmied, L., and Graner, A. (2006) Genetic mapping and BAC assignment of EST-derived SSR markers shows non-uniform distribution of genes in the barley genome. *Theor. Appl. Genet.*, **113**, 239–250.

66 Madishetty, K., Condamine, P., Svensson, J.T., Rodriguez, E., and Close, T.J. (2007) An improved method to identify BAC clones using pooled Overgos. *Nucleic Acids Res.*, **35** (1), e5. doi: 10.1093/nar/gkl920

67 http://www.ipk-gatersleben.de/Internet/Forschung/Projekte/Barley (8 February 2011).

68 Schulte, D., Ariyadasa, R., Poursarebani, N., Zhou, R., Sretenovic-Rajicic, T., Langridge, P., Shi, B.J., Mayer, K., Close, T., Weise, S., Scholtz, U., Graner, A., and Stein, S. (2010) Whole genome physical map of barley (*Hordeum vulgare* L.). W343, Plant and Animal Genome XVIII Conference, January 9–13, San Diego.

69 Bolot, S., Abrouk, M., Masood-Quraishi, U., Stein, N., Messing, J., Feuillet, C., and Salse, J. (2009) The "inner circle" of the cereal genomes. *Curr. Opin. Plant Biol.*, **12** (2), 119–125.

70 Mayer, K.F.X., Taudien, S., Martis, M., Imkova, H., Suchankova, P., Gundlach, H., Wicker, T., Petzold, A., Felder, M., Steuernagel, B., Scholz, U., Graner, A., Platzer, M., Doleel, J., and Stein, N. (2009) Gene content and virtual gene order of barley chromosome 1H. *Plant Physiol.*, **151**, 496–505.

71 Tondelli, A., Francia, E., Barabaschi, D., Pasquariello, M., and Pecchioni, N. (2011) Inside the CBF locus in *Poaceae*. *Plant Sci.*, **180**, 39–45.

72 Bevan, M.W., Garvin, D.F., and Vogel, J.P. (2010) *Brachypodium distachyon* genomics for sustainable food and fuel production. *Curr. Opin. Biotechnol.*, **21**, 211–217.

73 The International Brachypodium Initiative (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature*, **463**, 763–768.

74 Draper, J., Mur, L.A.J., Jenkins, G., Ghosh-Biswas, G.C., Bablak, P., Hasterok, R., and Routledge, A.P.M. (2001) *Brachypodium distachyon*: a new

model system for functional genomics in grasses. *Plant Physiol.*, **127**, 1539–1555.

75 Garvin, D.F. (2007) Brachypodium *distachyon*: a new model system for structural and functional analysis of grass genomes, in *Model Plants and Crop Improvement* (eds R.K. Varshney and R.M.D. Koebner), CRC Press, Boca Raton, pp. 109–124.

76 Vogel, J.P. and Hill, T. (2008) High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3. *Plant Cell Rep.*, **27**, 471–478.

77 Hong, S.J., Seo, P.J., Yang, M.S., Xiang, F., and Park, C.M. (2008) Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. *BMC Plant Biol.*, **8**, 112.

78 Luo, N., Liua, J., Yub, X., and Jiang, Y. (2011) Natural variation of drought response in *Brachypodium distachyon*. *Physiol. Plant.*, **141**, 19–29.

79 Rodriguez, M., Canales, E., and Borras-Hidalgo, O. (2005) Molecular aspects of abiotic stress in plants. *Biotechnol. Appl.*, **22**, 1–10.

80 Ramachandran, S. and Sundaresan, V. (2001) Transposons as tools for functional genomics. *Plant Physiol. Biochem.*, **39** (3–4), 243–252.

81 Harwood, W.A., Bartlett, J.G., Alves, S.C., Perry, M., Smedley, M.A., Layland, N., and Snape, J.W. (2009) Barley transformation using Agrobacterium-mediated techniques. *Methods Mol. Biol.*, **478**, 137–147.

82 Kumlehn, J., Serazetdinova, L., Hensel, G., Becker, D., and Loerz, H. (2006) Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. *Plant Biotechnol. J.*, **4** (2), 251–261.

83 Koprek, T., McElroy, D., Louwerse, J., Williams-Carrier, R., and Lemaux, P.G. (2000) An efficient method for dispersing Ds elements in the barley genome as a tool for determining gene function. *Plant J.*, **24**, 253–263.

84 Scholz, S., Lorz, H., and Lutticke, S. (2001) Transposition of the maize transposable element *Ac* in barley (*Hordeum vulgare* L.). *Mol. Genet. Genomics*, **264**, 653–661.

85 Mochida, K. and Shinozaki, K. (2010) Genomics and bioinformatics resources for crop improvement. *Plant Cell Physiol.*, **51** (4), 497–523.

86 Ayliffe, M.A., Pallotta, M., Langridge, P., and Pryor, A.J. (2007) A barley activation tagging system. *Plant Mol. Biol.*, **64**, 329–347.

87 Singh, J., Zhang, S., Chen, C., Cooper, L., Bregitzer, P., Sturbaum, A., Hayes, P.M., and Lemaux, P.G. (2006) High-frequency *Ds* remobilization over multiple generations in barley facilitates gene tagging in large genome cereals. *Plant Mol. Biol.*, **62**, 937–950.

88 Zhao, T., Pallotta, M., Langridge, P., Prasad, M., Graner, A., Schulze-Lefert, P., and Koprek, T. (2006) Mapped *Ds*/T-DNA launch pads for functional genomics in barley. *Plant J.*, **47**, 811–826.

89 Tester, M. and Bacic, A. (2005) Abiotic stress tolerance in grasses: from model plants to crop plants. *Plant Physiol.*, **137**, 791–793.

90 Vinocur, B. and Altman, A. (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr. Opin. Biotechnol.*, **16**, 123–132.

91 Morran, S., Eini, O., Pyvovarenko, T., Parent, B., Singh, R., Ismagul, A., Eliby, S., Sfirley, N., Langridge, P., and Lopato, S. (2011) Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. *Plant Biotechnol. J.*, **9**, 230–249.

92 Delhaize, E., Ryan, P.R., Hebb, D.M., Yamamoto, Y., Sasaki, T., and Matsumoto, H. (2004) Engineering high-level aluminum tolerance in barley with the *ALMT1* gene. *Proc. Natl. Acad. Sci. USA*, **202** (42), 15249–15254.

93 Xu, D., Duna, X., Wang, B., Hong, B., Ho, T.H.D., and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.*, **110** (1), 249–257.

94 Babu, R.C., Zhang, J., Blum, A., Ho, T.H.D., We, R., and Nguyen, H.T. (2004)

HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. *Plant Sci.*, **166**, 855–862.

95 Oh, S.J., Kwon, C.W., Choi, D.W., Song, S.L., and Kim, J.K. (2007) Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice. *Plant Biotechnol. J.*, **5** (5), 646–656.

96 Dong, W., Nowara, D., and Schweizer, P. (2006) Protein polyubiquitination plays a role in basal host resistance of barley. *Plant Cell*, **18**, 3321–3331.

97 Shen, Q.H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I.E., and Schulze-Lefert, P. (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science*, **315** (5815), 1098–1103.

98 Douchkov, D., Nowara, D., Zierold, U., and Schweizer, P. (2005) A high-throughput gene-silencing system for the functional assessment of defense related genes in barley epidermal cells. *Mol. Plant Microbe Interact.*, **18**, 755–761.

99 Marzin, S., Mihaly, R., Pauk, J., and Schweizer, P. (2008) A transient assay system for the assessment of cell autonomous gene function in dehydration-stressed barley. *J. Exp. Bot.*, **59** (12), 3359–3369.

100 Lazarow, K. and Lutticke, S. (2009) An Ac/Ds-mediated gene trap system for functional genomics in barley. *BMC Genomics*, **10**, 55.

101 Vagner, A.B., Visser, P.B., Angenent, G.C., and Krens, F.A. (2004) The potential of virus-induced gene silencing for speeding up the functional characterization of plant genes. *Genet. Mol. Res.*, **3** (3), 323–341.

102 Cogoni, C. and Macino, G. (1999) Homology dependent gene silencing in plants and fungi: a number of variations on the same theme. *Curr. Opin. Microbiol.*, **2** (6), 657–662.

103 Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G., and Waterhouse, P.M. (2000) Total silencing by intron-spliced hairpin RNAs. *Nature*, **407**, 319–320.

104 Wang, M.B., Abbott, D.C., and Waterhouse, P.M. (2000) A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. *Mol. Plant Pathol.*, **1** (6), 347–356.

105 Guo, H.S., Fei, J.F., Xie, Q., and Chua, N.H. (2003) A chemical-regulated inducible RNAi system in plants. *Plant J.*, **34**, 383–392.

106 Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M. (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. *Plant J.*, **34**, 733–739.

107 Ogo, Y., Kobayashi, T., Nakanishi Itai, R., Nakanishi, H., Kakei, Y., Takahashi, M., Toki, S., Mori, S., and Nishizawa, N.K. (2008) A novel NAC transcription factor, IDEF2, that recognizes the iron deficiency-responsive element 2 regulates the genes involved in iron homeostasis in plants. *J. Biol. Chem.*, **283** (19), 13407–13417.

108 Gery, C., Zuther, E., Schulz, E., Legoupi, J., Chauveau, A., McKhann, H., Hincha, D.K., and Téoulé, E. (2011) Natural variation in the freezing tolerance of *Arabidopsis thaliana*: effects of RNAi-induced CBF depletion and QTL localisation vary among accessions. *Plant Sci.*, **180**, 12–23.

109 Demircan, T. and Akkaya, M.S. (2010) Virus induced gene silencing in *Brachypodium distachyon:* a model organism for cereals. *Plant Cell Tissue Organ Cult.*, **100**, 91–96.

110 Holzberg, S., Brosio, P., Gross, C., and Pogue, G.P. (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J.*, **30**, 315–327.

111 Oikawa, A., Rahman, A., Yamashita, T., Taira, H., and Kidou, S. (2007) Virus-induced gene silencing of P23k in barley leaf reveals morphological changes involved in secondary wall formation. *J. Exp. Bot.*, **58**, 2617–2625.

112 Hein, I., Barciszewska-Pacak, M., Hrubikova, K., Williamson, S., Dinesen, M., Soenderby, I.E., Sundar, S., Jarmolowski, A., Shirasu, K., and Lacomme, C. (2005) Virus-induced gene

silencing-vased functional characterization of genes associated with powdery mildew resistance in barley. *Plant Physiol.*, **138**, 2155–2164.

113 Caldwell, D.G., McCallum, N., Shaw, P., Muehlbauer, G.J., Marshall, D.F., and Waugh, R. (2004) A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare* L.). *Plant J.*, **40** (1), 143–150.

114 Gottwald, S., Bauer, P., Komatsuda, T., Lundqvist, U., and Stein, N. (2009) TILLING in the two-rowed barley cultivar "Barke" reveals preferred sites of functional diversity in the gene HvHox1. *BMC Res. Note*, **2**, 258.

115 http://www.distagenomics.unibo.it/TILLMore/ (8 February 2011).

116 Talamè, V., Bovina, R., Sanguineti, M.C., Tuberosa, R., Lundqvist, U., and Salvi, S. (2008) TILLMore, a resource for the discovery of chemically induced mutants in barley. *Plant Biotechnol. J.*, **6** (5), 477–485.

117 Lababidi, S., Mejlhede, N., Rasmussen, S.K., Backes, G., Al-Said, W., Baum, M., and Jahoor, A.J. (2009) Identification of barley mutants in the cultivar "Lux" at the Dhn loci through TILLING. *Plant Breed.*, **128**, 332–336.

118 Wang, Z., Gerstein, M., and Snyder, M. (2009) RNA-seq: a revolutionary tool for transcriptomics. *Nat. Rev.*, **10**, 57–63.

119 Wang, L., Li, P., and Brutnell, P. (2010) Exploring plant transcriptomes using ultra high-throughput sequencing. *Brief. Funct. Genomics*, **9** (2), 118–128.

120 Close, T.J., Wanamaker, S.I., Caldo, R.A., Turner, S.M., Ashlock, D.A., Dickerson, J.A., Wing, R.A., Muehlbauer, G.J., Kleinhofs, A., and Wise, R.P. (2004) A new resource for cereal genomics: 22k barley GeneChip comes of age. *Plant Physiol.*, **134**, 960–968.

121 Wise, R., Caldo, R., Hong, L., Shen, L., Cannon, E., and Dickerson, J. (2007) BarleyBase/PLEXdb: a unified expression profiling database for plants and plant pathogens, in *Plant Bioinformatics: Methods and Protocols* (ed. D. Edwards), Methods in Molecular Biology, Humana Press, Totowa, pp. 347–363.

122 Schreiber, A.W., Sutton, T., Caldo, R.A., Kalashyan, E., Lovell, B., Mayo, G., Muehlbauer, G., Druka, A., Waugh, R., Wise, R., Langridge, P., and Baumann, U. (2009) Comparative transcriptomics in the *Triticeae*. *BMC Genomics*, **10**, 285.

123 Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., and Zimmermann, P. (2008) Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics*. doi: 10.1155/2008/420747

124 Leymarie, J., Bruneaux, E., Gibot-Leclerc, S., and Corbineau, F. (2007) Identification of transcripts potentially involved in barley seed germination and dormancy using cDNA-AFLP. *J. Exp. Bot.*, **58** (3), 425–437.

125 White, J., Pacey-Miller, T., Crawford, A., Cordeiro, G., Barbary, D., Bundock, P., and Henry, R. (2006) Abundant transcripts of malting barley identified by serial analysis of gene expression (SAGE). *Plant Biotechnol. J.*, **4**, 289–301.

126 Fischer, A., Lenhard, A., Tronecker, H., Lorat, Y., Kraenzle, M., Sorgenfrei, O., Zeppenfeld, T., Haushalter, M., Vogt, G., Gruene, U., Meyer, A., Handlbichler, U., Schweizer, P., and Gaelweiler, L. (2007) iGentifier: indexing and large-scale profiling of unknown transcriptomes. *Nucleic Acids Res.*, **35** (14), 4640–4648.

127 Barbazuk, W.B., Emrich, S.J., Chen, H.D., Li, L., and Schnable, P.S. (2007) SNP discovery via 454 transcriptome sequencing. *Plant J.*, **51** (5), 910–918.

128 Weber, A.P.M., Weber, K.L., Carr, K., Wilkerson, C., and Ohlrogge, J.B. (2007) Sampling the *Arabidopsis* transcriptome with massively parallel pyrosequencing. *Plant Physiol.*, **144**, 32–42.

129 Cheung, F., Haas, B.J., Goldberg, S.M., May, G.D., Xiao, Y., and Town, C.D. (2006) Sequencing *Medicago truncatula* expressed sequenced tags using 454 Life Sciences technology. *BMC Genomics*, **7**, 272.

130 Guo, S., Zheng, Y., Joung, J.-G., Liu, S., Zhang, Z., Crasta, O.R., Sobral, B.W., Xu, Y., Huang, S., and Fei, Z. (2010) Transcriptome sequencing and comparative analysis of cucumber

flowers with different sex types. *BMC Genomics*, **11**, 384.

131 Severin, A.J., Woody, J.L., Bolon, Y.T., Joseph, B., Diers, B.W., Farmer, A.D., Muehlbauer, G.J., Nelson, R.T., Grant, D., Specht, J.E., Graham, M.A., Cannon, S.B., May, G.D., Vance, C.P., and Shoemaker, R.C. (2010) RNA-Seq atlas of *Glycine max*: a guide to the soybean transcriptome. *BMC Plant Biol.*, **10**, 160.

132 Thakur, V. and Varshney, R. (2010) Challenges and strategies for next generation sequencing (NGS) data analysis. *J. Comput. Sci. Syst. Biol.*, **3** (2), 40–42.

133 http://www.public.iastate.edu/~imagefpc/IBSC%20Webpage/IBSC%20Template-publications.html (8 February 2011).

134 Faccioli, P., Pecchioni, N., Cattivelli, L., Stanca, A.M., and Terzi, V. (2001) Expressed sequence tags from cold-acclimatized barley can identify novel plant genes. *Plant Breed.*, **120**, 497–502.

135 Faccioli, P., Lagonigro, M.S., De Checco, L., Stanca, A.M., Alberici, R., and Terzi, V. (2002) Analysis of differential expression of barley ESTs during cold acclimatization using microarray technology. *Plant Biol.*, **4**, 630–639.

136 Atienza, S.G., Faccioli, P., Perrotta, G., Dalfino, G., Zschiesche, W., Humbeck, K., Stanca, A.M., and Cattivelli, L. (2004) Large scale analysis of transcripts abundance in barley subjected to several single and combined abiotic stress conditions. *Plant Sci.*, **167**, 1359–1365.

137 Tommasini, L., Svensson, J.T., Rodriguez, E.M., Wahid, A., Malatrasi, M., Kato, K., Wanamaker, S., Resnik, J., and Close, T.J. (2008) Dehydrin gene expression provides an indicator of low temperature and drought stress: transcriptome-based analysis of barley (*Hordeum vulgare* L.). *Funct. Integr. Genomics*, **8**, 387–405.

138 Dal Bosco, C., Busconi, M., Govoni, C., Baldi, P., Stanca, A.M., Crosatti, C., Bassi, R., and Cattivelli, L. (2003) *Cor* gene expression in barley mutants affected in chloroplast development and photosynthetic electron transport. *Plant Physiol.*, **131**, 793–802.

139 Svensson, J.T., Crosatti, C., Campoli, C., Bassi, R., Stanca, A.M., Close, T.J., and Cattivelli, L. (2006) Transcriptome analysis of cold acclimation in barley *Albina* and *Xantha* mutants. *Plant Physiol.*, **141**, 257–270.

140 Talamè, V., Ozturk, N.Z., Bohnert, H.J., and Tuberosa, R. (2007) Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. *J. Exp. Bot.*, **58** (2), 229–240.

141 Ozturk, Z.N., Talamè, V., Deyholos, M., Michalowski, C.B., Galbraith, D.W., Gozukirmizi, N., Tuberosa, R., and Bohnert, H.J. (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Mol. Biol.*, **48** (5–6), 551–573.

142 Close, T.J. (2005) The barley microarray: a community vision and application to abiotic stress. *Czech J. Genet. Plant Breed.*, **41** (4), 144–152.

143 Suprunova, T., Krugman, T., Distelfeld, A., Fahima, T., Nevo, E., and Korol, A. (2007) Identification of a novel gene (Hsdr4) involved in water-stress tolerance in wild barley. *Plant Mol. Biol.*, **64**, 17–34.

144 Guo, P., Baum, M., Grando, S., Ceccarelli, S., Bai, G., Li, R., von Korff, M., Graner, A., and Valcoun, J. (2009) Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. *J. Exp. Bot.*, **60**, 3531–3544.

145 Abebe, T., Melmaiee, K., Berg, V., and Wise, R.P. (2010) Drought response in the spikes of barley: gene expression in the lemma, palea, awn, and seed. *Funct. Integr. Genomics*, **10**, 191–205.

146 Ueda, A., Shi, W., Nakamura, T., and Takabe, T. (2002) Analysis of salt-inducible genes in barley roots by differential display. *J. Plant Res.*, **115**, 119–130.

147 Ueda, A., Kathiresan, A., Inada, M., Narita, Y., Nakamura, T., Shi, W., Takabe, T., and Bennett, J. (2004) Osmotic stress in barley regulates expression of a

different set of genes than salt stress does. *J. Exp. Bot.*, **55** (406), 2213–2218.

148 Walia, H., Wilson, C., Wahid, A., Condamine, P., Cui, X., and Close, T.J. (2006) Expression analysis of barley (*Hordeum vulgare* L.) during salinity stress. *Funct. Integr. Genomics*, **6**, 143–156.

149 Walia, H., Wilson, C., Condamine, P., Ismail, A.M., Xu, J., Cui, X., and Close, T.J. (2007) Array-based genotyping and expression analysis of barley cv. Maythorpe and Golden Promise. *BMC Genomics*, **8**, 87.

150 Colmer, T.D., Munns, R., and Flowers, T.J. (2005) Improving salt tolerance of wheat and barley: future prospects. *Aust. J. Exp. Agr.*, **45**, 1425–1443.

151 Ueda, A., Kathiresan, A., Bennett, J., and Takabe, T. (2006) Comparative transcriptome analyses of barley and rice under salt stress. *Theor. Appl. Genet.*, **112**, 1286–1294.

152 Walia, H., Wilson, C., Ismail, A.M., Close, T.J., and Cui, X. (2009) Comparing genomic expression patterns across plant species reveals highly diverged transcriptional dynamics in response to salt stress. *BMC Genomics*, **10**, 398.

153 Rabie, E. (1999) Altered nitrogen metabolism under environmental stress conditions, in *Handbook of Plant and Crop Stress*, 2nd edn (ed. M. Pessarakli), Marcel Dekker Inc., New York, pp. 349–363.

154 Wang, R., Guegler, K., LaBrie, S.T., and Crawforda, N.M. (2000) Genomic analysis of a nutrient response in *Arabidopsis* reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *Plant Cell*, **12**, 1491–1509.

155 Wang, Y.H., Garvin, D.F., and Kochian, L.V. (2001) Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. *Plant Physiol.*, **127** (1), 345–359.

156 Kumar, R., Taware, R., Gaur, V.S., Guru, S.K., and Kumar, A. (2009) Influence of nitrogen on the expression of *TaDof1* transcription factor in wheat and its relationship with photosynthetic and ammonium assimilating efficiency. *Mol. Biol. Rep.*, **36** (8), 2209–2220.

157 Trueman, L.J., Richardson, A., and Forde, B.G. (1996) Molecular cloning of higher plant homologues of the high-affinity nitrate transporters of *Chlamydomonas reinhardtii* and *Aspergillus nidulans*. *Gene*, **175**, 223–231.

158 Vidmar, J.J., Zhuo, D., Siddiqi, M.Y., and Glass, A.D.M. (2000) Isolation and characterization of *HvNRT2.3* and *HvNRT2.4*, cDNAs encoding high-affinity nitrate transporters from roots of barley. *Plant Physiol.*, **122**, 783–792.

159 Wang, C., van den Ende, W., and Tillberg, J.E. (2000) Fructan accumulation induced by nitrogen deficiency in barley leaves correlates with the level of sucrose: fructan 6-fructosyltransferase mRNA. *Planta*, **211** (5), 701–707.

160 Morcuende, R., Bari, R., Gibon, Y., Zheng, W.M., Pant, B.D., Blasing, O., Usadel, B., Czechowski, T., Udvardi, M.K., Stitt, M., and Scheible, W.R. (2007) Genome-wide reprogramming of metabolism and regulatory networks of *Arabidopsis* in response to phosphorus. *Plant Cell Environ.*, **30**, 85–112.

161 Wasaki, J., Yonetani, R., Kuroda, S., Shinano, T., Yazaki, J., Fujii, F., Shimbo, K., Yamamoto, K., Sakata, K., Sasaki, T., Kishimoto, N., Kikuchi, S., Yamagishi, M., and Osaki, M. (2003) Transcriptomic analysis of metabolic changes by phosphorus stress in rice plant roots. *Plant Cell Environ.*, **26**, 1515–1523.

162 Uhde-Stone, C., Zinn, K.E., Ramirez-Yanez, M., Li, A.G., Vance, C.P., and Allan, D.L. (2003) Nylon filter arrays reveal differential gene expression in proteoid roots of white lupin in response to phosphorus deficiency. *Plant Physiol.*, **131**, 1064–1079.

163 Furukawa, J., Yamaji, N., Wang, H., Mitani, N., Murata, Y., Sato, K., Katsuhara, M., Takeda, K., and Ma, J.F. (2007) An aluminium-activated citrate transporter in barley. *Plant Cell Physiol.*, **38** (8), 1081–1091.

164 http://www.plexdb.org/modules/PD_browse/experiment_browser.php?experiment=BB83&genechip=Barley1 (8 February 2011).

165 Sutton, T., Baumann, U., Hyes, J., Collins, N.C., Shi, B.J., Schnurbusch, T., Hay, A., Mayo, G., Pallotta, M., Tester, M., and Langridge, P. (2007) Boron-toxicity tolerance in barley arising from efflux transporter amplification. *Science*, **318**, 1446–1449.

166 Oz, M.T., Yilmaz, R., Eyidogan, F., de Graaff, L., Yucel, M., and Oktemi, H.A. (2009) Microarray analysis of late response to boron toxicity in barley (*Hordeum vulgare* L.) leaves. *Turkish J. Agric. Forest.*, **33**, 191–202.

167 Suzuki, M., Takahashi, M., Tsukamoto, T., Watanabe, S., Matsuhashi, S., Yazaki, J., Kishimoto, N., Kikuchi, S., Nakanishi, H., Mori, S., and Nishizawa, N.K. (2006) Biosynthesis and secretion of mugineic acid family phytosiderophores in zinc-deficient barley. *Plant J.*, **48**, 85–97.

168 Richards, R.A. (1996) Defining selection criteria to improve yield under drought. *Plant Growth Regul.*, **20** (2), 157–166.

169 Lander, E.S. and Botstein, D. (1989) Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics*, **121**, 185–199.

170 Jansen, R.C. (1993) Interval mapping of multiple quantitative trait loci. *Genetics*, **135**, 205–211.

171 Zeng, Z.B. (1994) Precision mapping of quantitative trait loci. *Genetics*, **136**, 1457–1468.

172 Kraakman, A.T.W., Niks, R.E., van den Berg, P.M.M.M., Stam, P., and van Eeuwijk, F.A. (2004) Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. *Genetics*, **168**, 435–446.

173 Jansen, R.C., Tesson, B.M., Fu, J., Yang, Y., and McIntyre, L.M. (2009) Defining gene and QTL networks. *Curr. Opin. Plant Biol.*, **12**, 241–246.

174 Matthews, D.E., Carollo, V.L., Lazo, G.R., and Anderson, O.D. (2003) GrainGenes: the genome database for small-grain crops. *Nucleic Acids Res.*, **31**, 183–186.

175 Jaiswal, P., Ni, J., Yap, I., Ware, D., Spooner, W., Youens-Clark, K., Ren, L., Liang, C., Zhao, W., Ratnapu, K., Faga, B., Canaran, P., Fogleman, M., Hebbard, C., Avraham, S., Schmidt, S., Casstevens, T.M., Buckler, E.S., Stein, L., and McCouch, S. (2006) Gramene: a bird's eye view of cereal genomes. *Nucleic Acids Res.*, **34** (Database Issue), D717–D723.

176 Milc, J., Sala, A., Bergamaschi, S., and Pecchioni, N. (2011) A genotypic and phenotypic information source for marker-assisted selection of cereals: the CEREALAB Database. *Database*. doi: 10.1093/database/baq038

177 Glass, G.V. (1976) Primary, secondary and meta-analysis of research. *Educ. Res.*, **5**, 3–8.

178 Goffinet, B. and Gerber, S. (2000) Quantitative trait loci: a meta-analysis. *Genetics*, **155**, 463–473.

179 Khowaja, F.S., Norton, G.J., Courtois, B., and Price, A.H. (2009) Improved resolution in the position of drought-related QTLs in a single mapping population of rice by meta-analysis. *BMC Genomics*, **10**, 276.

180 Cattivelli, L., Baldi, P., Crosatti, C., Di Fonzo, N., Faccioli, P., Grossi, M., Mastrangelo, A.M., Pecchioni, N., and Stanca, A.M. (2002) Chromosome regions and stress-related sequences involved in resistance to abiotic stress in *Triticeae*. *Plant Mol. Biol.*, **48** (5–6), 649–665.

181 Kleinhofs, A. and Graner, A. (2001) An integrated map of the barley genome, in *The DNA-Based Markers in Plants*, 2nd edn (eds R.L. Phillips and I.K. Vasil), Kluwer Academic Publishers, Dordrecht, pp. 187–199.

182 Vannini, C., Locatelli, F., Bracale, M., Magnani, E., Marsoni, M., Osnato, M., Mattana, M., Baldoni, E., and Coraggio, I. (2004) Overexpression of the rice *Osmyb4* gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. *Plant J.*, **37** (1), 115–127.

183 Casaretto, J. and Ho, T.D. (2003) The transcription factors HvABI5 and HvVP1 are required for the abscisic acid induction of gene expression in barley aleurone cells. *Plant Cell*, **15** (1), 271–284.

184 Kobayashi, F., Maeta, E., Terashima, A., and Takumi, S. (2008) Positive role of a wheat HvABI5 ortholog in abiotic stress response of seedlings. *Physiol. Plant.*, **134**, 74–86.

185 Price, A.H. (2006) Believe it or not, QTLs are accurate!. *Trends Plant Sci.*, **11** (5), 213–216.

186 Romagosa, I., Ullrich, S.E., Han, F., and Hayes, P.M. (1996) Use of the additive main effects and multiplicative interaction model in QTL mapping for adaptation in barley. *Theor. Appl. Genet.*, **93** (1–2), 30–37.

187 Xu, S. and Jia, Z. (2007) Genomewide analysis of epistatic effects for quantitative traits in barley. *Genetics*, **175**, 1955–1963.

188 Comadran, J., Russell, J.R., van Eeuwijk, F.A., Ceccarelli, S., Grando, S., Baum, M., Stanca, A.M., Pecchioni, N., Mastrangelo, A.M., Akar, T., Al-Yassin, A., Benbelkacem, A., Choumane, W., Ouabbou, H., Dahan, R., Bort, J., Araus, J.-L., Pswarayi, A., Romagosa, I., Hackett, C.A., and Thomas, W.T.B. (2008) Mapping adaptation of barley to droughted environments. *Euphytica*, **161** 35–45.

189 Francia, E., Rizza, F., Cattivelli, L., Stanca, A.M., Galiba, G., Toth, B., Hayes, P.M., Skinner, J.S., and Pecchioni, N. (2004) Two loci on chromosome 5H determine low-temperature tolerance in a "Nure" (winter) × "Tremois" (spring) barley map. *Theor. Appl. Genet.*, **108**, 670–680.

190 Jansen, R.C. and Nap, J.P. (2001) Genetical genomics: the added value from segregation. *Trends Genet.*, **17** (7), 388–391.

191 de Koning, D.J. and Haley, C.S. (2005) Genetical genomics in humans and model organisms. *Trends Genet.*, **21** (7), 377–381.

192 West, M.A.L., Leeuwen, H., Kozik, A., Kliebenstein, D.K., Doerge, R.W., Clair, D.A., and Michelmore, R.W. (2006) High-density haplotying with microarray-based expression and single feature polymorphism markers in *Arabidopsis*. *Genome Res.*, **16**, 787–795.

193 Wang, J., Yu, H., Xie, W., Xing, Y., Yu, S., Xu, C., Li, X., Xiao, J., and Zhang, Q. (2010) A global analysis of QTLs for expression variations in rice shoots at the early seedling stage. *Plant J.*, **63**, 1063–1074.

194 Chen, X., Hackett, C.A., Niks, R.E., Hedley, P.E., Booth, C., Druka, A., Marcel, T.C., Vels, A., Bayer, M., Milne, I., Morris, J., Ramsay, L., Marshall, D., Cardle, L., and Waugh, R. (2010) An eQTL analysis of partial resistance to *Puccinia hordei* in barley. *PLoS ONE*, **5** (1), e8598.

195 Hays, D., Mason, E., Hwa Do, J., Menz, M., and Reynolds, M. (2007) Expression quantitative trait loci mapping heat tolerance during reproductive development in wheat (*Triticum aestivum*), in *Wheat Production in Stressed Environments* (eds H.T. Buck, J.E. Nisi, and N. Salomón), Springer, Amsterdam, pp. 373–382.

196 Jordan, M.C. and Banks, T.W. (2011) Integration of array-based markers and expression QTL with genetic maps in wheat. P274. Plant and Animal Genomes XIX Conference, Jan. 15–19, 2011, San Diego, CA.

197 Witzel, K., Pietsch, C., Strickert, M., Matros, A., Röder, M.S., Weschke, W., Wobus, U., and Mock, H. (2010) Mapping of quantitative trait loci associated with protein expression variation in barley grains. *Mol. Breed.* doi: 10.1007/s11032-010-9432-2

198 Meyer, R.C., Steinfath, M., Lisec, J., Becher, M., Witucka-Wall, H., Torjék, H., Fiehn, O., Eckardt, A., Willmitzer, L., Selbig, J., and Altmann, T. (2007) The metabolic signature related to high plant growth rate in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, **104**, 4759–4764.

199 Rowe, H.C., Hansen, B.G., Halkier, B.A., and Kliebenstein, D.J. (2008) Biochemical networks and epistasis shape the *Arabidopsis thaliana* metabolome. *Plant Cell*, **20**, 1199–1216.

200 West, M.A.L., Kim, K., Kliebenstein, D.J., van Leeuwen, H., Michelmore, R.W., Doerge, R.W., and St Clair, D.A. (2007) Global eQTL mapping reveals the complex genetic architecture of transcript level variation in *Arabidopsis*. *Genetics*, **175**, 1441–1450.

201 Rapacz, M., Wolanin, B., Hura, K., and Tyrka, M. (2008) The effects of cold acclimation on photosynthetic apparatus

and the expression of COR14b in four genotypes of barley (*Hordeum vulgare*) contrasting in their tolerance to freezing and high-light treatment in cold conditions. *Ann. Bot.*, **101**, 689–699.

202 Sutton, F., Chen, D.G., Ge, X., and Kenefick, D. (2009) *Cbf* genes of the *Fr-A2* allele are differentially regulated between long-term cold acclimated crown tissue of freeze-resistant and susceptible, winter wheat mutant lines. *BMC Plant Biol.*, **9**, 34.

203 Hayes, P.M., Blake, T., Chen, T.H.H., Tragoonrung, S., Chen, F., Pan, A., and Liu, B. (1993) Quantitative trait loci on barley (*Hordeum vulgare* L.) chromosome 7 associated with components of winter hardiness. *Genome*, **36**, 66–71.

204 Pan, A., Hayes, P.M., Chen, F., Blake, T., Wright, S., Karsai, I., and Bedo, Z. (1994) Genetic analysis of the components of winter hardiness in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, **89**, 900–910.

205 Galiba, G., Quarrie, S.A., Sutka, J., Morgounov, A., and Snape, J.W. (1995) RFLP mapping of the vernalization (*Vrn1*) and frost resistance (*Fr1*) genes on chromosome 5 A of wheat. *Theor. Appl. Genet.*, **90** (7–8), 1174–1179.

206 Vagujfalvi, A., Galiba, G., Cattivelli, L., and Dubcovsky, J. (2003) The cold-regulated transcriptional activator Cbf3 is linked to the frost-tolerance locus Fr-A2 on wheat chromosome 5A. *Mol. Genet. Genomics*, **269** (1), 60–67.

207 Galiba, G., Pecchioni, N., Vágújfalvi, A., Francia, E., Toth, B., Barabaschi, D., Barilli, S., Crosatti, C., Cattivelli, L., and Stanca, A.M. (2005) Localization of QTLs and candidate genes involved in the regulation of frost resistance in cereals. Proceedings of the International Congress "In the Wake of the Double Helix: From the Green Revolution to the Gene Revolution," 27–31 May 2003, Bologna, pp. 253–266.

208 Skinner, J., Szucs, P., von Zitzewitz, J., Marquez-Cedillo, L., Filichkin, T., Stockinger, E.J., Thomashow, M.F., Chen, T.H.H., and Hayes, P.M. (2006) Mapping of barley homologs to genes that regulate low temperature tolerance in *Arabidopsis*. *Theor. Appl. Genet.*, **112**, 832–842.

209 Francia, E., Barabaschi, D., Tondelli, A., Laidò, G., Rizza, F., Stanca, A.M., Busconi, M., Fogher, C., Stockinger, E.J., and Pecchioni, N. (2007) Fine mapping of a *HvCBF* gene cluster at the frost resistance locus *Fr-H2* in barley. *Theor. Appl. Genet.*, **115**, 1083–1091.

210 Knox, A.K., Dhillon, T., Cheng, H., Tondelli, A., Pecchioni, N., and Stockinger, E.J. (2010) *CBF* gene copy number variation at *Frost Resistance-2* is associated with levels of freezing tolerance in temperate-climate cereals. *Theor. Appl. Genet.*, **121**, 21–35.

211 Xiong, L., Lee, H., Ishitani, M., Lee, H., Zhang, C., and Zhu, J.K. (2001) *FIERY1* encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in *Arabidopsis*. *Gene Dev.*, **15**, 1971–1984.

212 Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B., Hong, X., Agarwal, A., and Zhu, J.K. (2003) *ICE1*: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Gene Dev.*, **17**, 1043–1054.

213 von Zitzewitz, J., Szucs, P., Dubcovsky, J., Yan, L., Francia, E., Pecchioni, N., Casas, A., Chen, T.H.H., Hayes, P.M., and Skinner, J.S. (2005) Molecular and structural characterization of barley vernalization genes. *Plant Mol. Biol.*, **59**, 449–467.

214 Cooper, L.L.D., von Zitzewitz, J., Skinner, J.S., Szucs, P., Karsai, I., Francia, E., Stanca, A.M., Pecchioni, N., Laurie, D.A., Chen, T.H.H., and Hayes, P.M. (2006) The genetic basis of vernalization response in barley, in *Advances in Plant Cold Hardiness* (ed. T.H.H. Chen), CABI Publishing, Wallingford, Oxfordshire, pp. 64–75.

215 Limin, A.E. and Fowler, D.B. (2006) Low-temperature tolerance and genetic potential in wheat (*Triticum aestivum* L.): response to photoperiod, vernalization, and plant development. *Planta*, **224**, 360–366.

216 Galiba, G., Vagujfalvi, A., Li, C., Soltesz, A., and Dubcovsky, J. (2009) Regulatory genes involved in the

determination of frost tolerance in temperate cereals. *Plant Sci.*, **176**, 12–19.

217 Stockinger, E.J., Skinner, J.S., Gardner, K.G., Francia, E., and Pecchioni, N. (2007) Expression levels of barley Cbf genes at the *Frost resistance-H2* locus are dependent upon alleles at *Fr-H1* and *Fr-H2*. *Plant J.*, **51** (2), 308–321.

218 Dhillon, S.P., Pearce, E.J., Stockinger, A., Distelfeld, C., Li, A.K., Knox, I., Vashegyi, A., Vagujfalvi, G., Galiba, J., and Dubcovsky, T. (2010) Regulation of freezing tolerance and flowering in temperate cereals: the *VRN-1* connection. *Plant Physiol.*, **153**, 1846–1858.

219 Chen, A., Baumann, U., Fincher, G.B., and Collins, N.C. (2009) *Flt-2L*, a locus in barley controlling flowering time, spike density, and plant height. *Funct. Integr. Genomics*, **9** (2), 243–254.

220 Pennisi, E. (2008) Plant genetics: the blue revolution, drop by drop, gene by gene. *Science*, **320** (5873), 171–173.

221 Maccaferri, M., Sanguineti, M.C., Giuliani, S., and Tuberosa, R. (2009) Genomics of tolerance to abiotic stress in the triticecae, in *Genetics and Genomics of the Triticeae* (eds G.J. Muehlbauer and C. Feuillet), Springer Science + Business Media, pp. 481–558.

222 Jones, H.G. (2007) Monitoring plant and soil water status: established and novel methods revisited and their relevance to studies of drought tolerance. *J. Exp. Bot.*, **58**, 119–130.

223 Pswarayi, A., van Eeuwijk, F.A., Ceccarelli, S., Grando, S., Comadran, J., Russell, J.R., Francia, E., Pecchioni, N., Li Destri, O., Akar, T., Al-Yassin, A., Benbelkacem, A., Choumane, W., Karrou, M., Ouabbou, H., Bort, J., Araus, J.L., Molina-Cano, J.L., Thomas, W.T.B., and Romagosa, I. (2008) Barley adaptation and improvement in the Mediterranean basin. *Plant Breed.*, **127**, 554–560.

224 Richards, R.A. (2006) Physiological traits used in the breeding of new cultivars for water-scarce environments. *Agr. Water Manage.*, **80** (1–3), 197–211.

225 Francia, E., Tondelli, A., Rizza, F., Badeckc, F.W., Li Destri Nicosia, O., Akar, T., Grando, S., Al-Yassin, A., Benbelkacem, A., Thomas, W.T.B., van Eeuwijk, F., Romagosa, I., Stanca, A.M., and Pecchioni, N. (2011) Determinants of barley grain yield in a wide range of Mediterranean environments. *Field Crops Res.*, **120** (1), 169–178.

226 Diab, A.A., Teulat-Merah, B., This, D., Ozturk, N.Z., Benscher, D., and Sorrells, M.E. (2004) Identification of drought-inducible genes and differentially expressed sequence tags in barley. *Theor. Appl. Genet.*, **109** (7), 1417–1425.

227 Teulat, B., Merah, O., Sirault, X., Borries, C., Waugh, R., and This, D. (2002) QTL for grain carbon isotope discrimination in field-grown barley. *Theor. Appl. Genet.*, **106**, 118–126.

228 Morandini, P. and Salamini, F. (2003) Plant biotechnology and breeding: allied for years to come. *Trends Plant Sci.*, **8** (2), 70–75.

229 Chen, G., Krugman, T., Fahima, T., Chen, K., Hu, Y., Roder, M., Nevo, E., and Korol, A. (2010) Chromosomal regions controlling seedling drought resistance in Israeli wild barley, *Hordeum spontaneum* C. Koch. *Genet. Resour. Crop. Evol.*, **57** (1), 85–99.

230 Teulat, B., Borries, C., and This, D. (2001) New QTLs identified for plant water status, water-soluble carbohydrate and osmotic adjustment in a barley population grown in a growth chamber under two water regimes. *Theor. Appl. Genet.*, **103** (1), 161–170.

231 Teulat, B., Zoumarou-Wallis, N., Rotter, B., Ben Salem, M., Bahri, H., and This, D. (2003) QTL for relative water content in fieldgrown barley and their stability across Mediterranean environments. *Theor. Appl. Genet.*, **108** (1), 181–188.

232 Mano, Y. and Takeda, K. (1997) Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.). *Euphytica*, **94**, 263–272.

233 Munns, R. and Rawson, H.M. (1999) Effect of salinity on salt accumulation and reproductive development in the apical meristem of wheat and barley. *Aust. J. Plant Physiol.*, **26** (5), 459–464.

234 Ellis, R.P., Forster, B.P., Gordon, D.C., Handley, L.L., Keith, R.P., Lawrence, P., Meyer, R., Powell, W., Robinson, D., Scrimgeour, C.M., Young, G., and Thomas, W.T.B. (2002) Phenotype/genotype associations for yield and salt tolerance in a barley mapping population segregating for two dwarfing genes. *J. Exp. Bot.*, **53** (371), 1163–1176.

235 Forster, B.P., Phillips, M.S., Miller, T.E., Baird, E., and Powell, W. (1990) Chromosome location of genes controlling tolerance to salt (NaCl) and vigour in *Hordeum vulgare* and *H. chilense*. *Heredity*, **65**, 99–107.

236 Witzel, K., Weidner, A., Surabhi, G., Varshney, R.K., Kunze, G., Buck-Sorlin, G.H., Borner, A., and Mock, H. (2010) Comparative analysis of the grain proteome fraction in barley genotypes with contrasting salinity tolerance during germination. *Plant Cell Environ.*, **33** (2), 211–222.

237 Raun, W.R. and Johnson, G.V. (1999) Improving nitrogen use efficiency for cereal production. *Agron. J.*, **91**, 357–363.

238 Mickelson, S., See, D., Meyer, F.D., Garner, J.P., Foster, C.R., Blake, T.K., and Fischer, A.M. (2003) Mapping of QTL associated with nitrogen storage and remobilization in barley (*Hordeum vulgare* L.) leaves. *J. Exp. Bot.*, **54** (383), 801–812.

239 Coque, M. and Gallais, A. (2006) Genomic regions involved in response to grain yield selection at high and low nitrogen fertilization in maize. *Theor. Appl. Genet.*, **112**, 1205–1220.

240 Gallais, A. and Hirel, B. (2004) An approach to the genetics of nitrogen use efficiency in maize. *J. Exp. Bot.*, **55** (396), 295–306.

241 Wissuwa, M., Gamat, G., and Ismail, A.M. (2005) Is root growth under phosphorus deficiency affected by source or sink limitations? *J. Exp. Bot.*, **56**, 1943–1950.

242 Minella, E. and Sorrells, M.E. (1997) Inheritance and chromosome location of *Alp*, a gene controlling aluminium tolerance in "Dayton" barley. *Plant Breed.*, **116** (5), 465–469.

243 Raman, H., Moroni, S., Sato, K., Read, J., and Scott, J. (2002) Identification of AFLP and microsatellite markers linked with an aluminium tolerance gene in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, **105**, 458–464.

244 Wang, J., Raman, H., Zhou, M., Ryan, P.R., Delhaize, E., Hebb, D.M., Coombes, N., and Mendham, N. (2007) High-resolution mapping of the Alp locus and identification of a candidate gene *HvMATE* controlling aluminium tolerance in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, **115** (2), 265–276.

245 Raman, H., Wang, J.P., Read, B., Zhou, M.X., Vengatanagappa, S., Moroni, J.S., O'Bree, B., and Mendham, N. (2005) Molecular mapping of resistance to aluminium toxicity in barley. Proceedings of Plant and Animal Genome XIII Conference, January 15–19, 2005, San Diego, pp. 154.

246 Navakode, S., Weidner, A., Varshney, R.K., Lohwasser, U., Scholz, U., and Borner, A. (2009) A QTL analysis of aluminium tolerance in barley using gene-based markers. *Cereal Res. Commun.*, **37** (4), 531–540.

247 Hayes, J.E. and Reid, R.J. (2004) Boron tolerance in barley is mediated by efflux of B from the roots. *Plant Physiol.*, **136**, 3376–3382.

248 Schnurbusch, T., Hayes, J., Hrmova, M., Baumann, U., Ramesh, S.A., Tyerman, S.D., Langridge, P., and Sutton, T. (2010) Boron toxicity tolerance in barley through reduced expression of the multifunctional aquaporin HvNIP2;1. *Plant Physiol.*, **153** (4), 1706–1715.

249 Chiba, Y., Mitani, N., Yamaji, N., and Ma, J.F. (2009) HvLsi1 is a silicon influx transporter in barley. *Plant J.*, **57** (5), 810–818.

250 Zhou, M., Li, H.B., and Mendham, N.J. (2007) Combining ability of waterlogging tolerance in barley (*Hordeum vulgare* L.). *Crop Sci.*, **47**, 278–284.

251 Setter, T.L. and Waters, I. (2003) Review of prospects for germplasm improvement for waterlogging tolerance in wheat, barley and oats. *Plant Soil.*, **253**, 1–33.

252 Boru, G., van Ginkel, M., Kronstad, W.E., and Boersma, L. (2001) Expression and

inheritance of tolerance to waterlogging stress in wheat. *Euphytica*, **117**, 91–98.
253 Reyna, N., Cornelious, B., Shannon, J.G., and Sneller, C.H. (2003) Evaluation of a QTL for waterlogging tolerance in Southern soybean germplasm. *Crop Sci.*, **43**, 2077–2082.
254 Li, H.B., Vaillancourt, R., Mendham, N.J., and Zhou, M.X. (2008) Comparative mapping of quantitative trait loci associated with waterlogging tolerance in barley (*Hordeum vulgare* L.). *BMC Genomics*, **9**, 401.
255 Zhou, M. (2010) Accurate phenotyping reveals better QTL for waterlogging tolerance in barley. *Plant Breed.* doi: 10.1111/j.1439-0523.2010.01792.x
256 Nanjo, Y., Nouri, M., and Komatsu, S. (2010) Quantitative proteomic analyses of crop seedlings subjected to stress conditions; a commentary. *Phytochemistry*. doi: 10.1016/j.phytochem.2010.10.017
257 Wang, W., Tai, F., and Chen, S. (2008) Optimizing protein extraction from plant tissues for enhanced proteomics analysis. *J. Sep. Sci.*, **31**, 2032–2039.
258 Grobei, M.A., Qeli, E., Brunner, E., Rehrauer, H., Zhang, R., Roschitzki, B., Basler, K., Ahrens, C.H., and Grossniklaus, U. (2009) Deterministic protein inference for shotgun proteomics data provides new insights into *Arabidopsis* pollen development and function. *Genome Res.*, **19**, 1786–1800.
259 Gstaiger, M. and Aebersold, R. (2009) Applying mass spectrometry-based proteomics to genetics, genomics and network biology. *Nat. Rev. Genet.*, **10**, 617–627.
260 Yates, J.R., Ruse, C.I., and Nakorchevsky, A. (2009) Proteomics by mass spectrometry: approaches, advances, and applications. *Ann. Rev. Biomed. Eng.*, **11**, 49–79.
261 Salekdeh, G.H. and Komatsu, S. (2007) Crop proteomics: aim at sustainable agriculture of tomorrow. *Proteomics*, **7**, 2976–2996.
262 Agrawal, G.K., Jwa, N.S., and Rakwal, R. (2009) Rice proteomics: ending phase I and the beginning of phase II. *Proteomics*, **9**, 935–963.
263 Komatsu, S. and Ahsan, N. (2009) Soybean proteomics and its application to functional analysis. *J. Proteomics*, **72**, 325–336.
264 Koller, A., Washburn, M.P., Lange, B.M., Andon, N.L., Deciu, C., Haynes, P.A., Hays, L., Schieltz, D., Ulaszek, R., Wei, J., Wolters, D., and Yates, J.R. (2002) Proteomic survey of metabolic pathways in rice. *Proc. Natl. Acad. Sci. USA*, **99**, 11969–11974.
265 Komatsu, S., Kojima, K., Suzuki, K., Ozaki, K., and Higo, K. (2004) Rice proteome database based on two-dimensional polyacrylamide gel electrophoresis: its status in 2003. *Nucleic Acids Res.*, **32**, D388–D392.
266 Finnie, C. and Svensson, B. (2009) Barley seed proteomics from spot to structures. *J. Proteomics*, **72**, 315–324.
267 Finnie, C., Bagge, M., Steenholdt, T., Ostergaard, O., Bak-Jensen, K.S., Backes, G., Jensen, A., Giese, H., Larsen, J., Roepstorff, P., and Svensson, B. (2009) Integration of the barley genetic and seed proteome maps for chromosome 1H, 2H, 3H, 5H and 7H. *Funct. Integr. Genomics*, **9**, 135–143.
268 Consoli, L., Lefevre, A., Zivy, M., de Vienne, D., and Damerval, C. (2002) QTL analysis of proteome and transcriptome variations for dissecting the genetic architecture of complex traits in maize. *Plant Mol. Biol.*, **48**, 575–581.
269 Neilson, K.A., Gammulla, C.G., Mirzaei, M., Imin, N., and Haynes, P.A. (2010) Proteomic analysis of temperature stress in plants. *Proteomics*, **10**, 828–845.
270 Majoul, T., Bancel, E., Triboi, E., Ben Hamida, J., and Branlard, G. (2003) Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from total endosperm. *Proteomics*, **3**, 175–183.
271 Lee, D.G., Ahsan, N., Lee, S.H., Kang, K.Y., Bahk, J.D., Lee, I.J., and Lee Dr, B.H. (2007) A proteomic approach in analyzing heat-responsive proteins in rice leaves. *Proteomics*, **7**, 3369–3383.
272 Sule, A., Vanrobaeys, F., Hajos, G., van Beeumen, J., and Devreese, B. (2004) Proteomic analysis of small heat shock

protein isoforms in barley shoots. *Phytochemistry*, **65**, 1853–1863.

273 Panjabi-Sabharwal, V., Karan, R., Khan, T., and Pareek, A. (2010) Abiotic stress responses: complexities in gene expression, in *Abiotic Stress Adaptation in Plants: Physiological, Molecular and Genomic Foundation* (eds A. Pareek, S.K. Sopory, and H.J. Bohnert), Springer, Dordrecht, pp. 177–198.

274 Hajheidari, M., Abdollahian-Noghabi, M., Askari, H., Heidari, M., Sadeghian, S.Y., Ober, E.S., and Salekdeh, G.H. (2005) Proteome analysis of sugar beet leaves under drought stress. *Proteomics*, **5**, 950–960.

275 Hajheidari, M., Eivazi, A., Buchanan, B.B., Wong, J.H., Majidi, I., and Salekdeh, G.H. (2007) Proteomics uncovers a role for redox in drought tolerance in wheat. *J. Proteome Res.*, **6**, 1451–1460.

276 Witzel, K., Weidner, A., Surabhi, G.K., Borner, A., and Mock, H.P. (2009) Salt stress-induced alterations in the root proteome of barley genotypes with contrasting response towards salinity. *J. Exp. Bot.*, **60**, 3545–3557.

277 Aghaei, K., Ehsanpour, A.A., Shah, A.H., and Komatsu, S. (2009) Proteome analysis of soybean hypocotyl and root under salt stress. *Amino Acids*, **36**, 91–98.

278 Lee, D.G., Ahsan, N., Lee, S.H., Lee, J.J., Bahka, J.D., Kanga, K.Y., and Lee, B.H. (2009) Chilling stress-induced proteomic changes in rice roots. *J. Plant Physiol.*, **166**, 1–11.

279 Laino, P., Shelton, D., Finnie, C., De Leonardis, A.M., Mastrangelo, A.M., Svensson, B., Lafiandra, D., and Masci, S. (2010) Comparative proteome analysis of metabolic proteins from seeds of durum wheat (cv. Svevo) subjected to heat stress. *Proteomics*, **10**, 2359–2368.

280 Patterson, J., Ford, K., Cassin, A., Natera, S., and Bacic, A. (2007) Increased abundance of proteins involved in phytosiderophore production in boron-tolerant barley. *Plant Physiol.*, **144**, 1612–1631.

281 Schneider, T., Schellenberg, M., Meyer, S., Keller, F., Gehrig, P., Riedel, K., Lee, Y., Eberl, L., and Martinoia, E. (2009) Quantitative detection of changes in the leaf-mesophyll tonoplast proteome in dependency of a cadmium exposure of barley (*Hordeum vulgare* L.) plants. *Proteomics*, **9**, 2668–2677.

282 Ahsan, N., Renaut, J., and Komatsu, S. (2009) Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. *Proteomics*, **9**, 2602–2621.

283 Hashiguchi, A., Ahsan, N., and Komatsu, S. (2010) Proteomics application of crops in the context of climatic changes. *Food Res. Intern.*, **43**, 1803–1813.

284 Bahrman, N., Gouy, A., Devienne-Barret, F., Hirel, B., Vedele, F., and Le Gouis, J. (2005) Differential change in root protein patterns of two wheat varieties under high and low nitrogen nutrition levels. *Plant Sci.*, **168**, 81–87.

285 Ding, C., You, J., Liu, Z., Rehmani, M., Wang, S., Li, G., Wang, Q., and Ding, Y. (2010) Proteomic analysis of low nitrogen stress-responsive proteins in roots of rice. *Plant Mol. Biol. Rep*. doi: 10.1007/s11105-010-0268-z

286 Bahrman, N., Le Gouis, J., Negroni, L., Amilhat, L., Leroy, P., Lainé, A.L., and Jaminon, O. (2004) Differential protein expression assessed by two-dimensional gel electrophoresis for two wheat varieties grown at four nitrogen levels. *Proteomics*, **4**, 709–719.

287 Song, C., Zeng, F., Feibo, W., Ma, W., and Zhang, G. (2010) Proteomic analysis of nitrogen stress-responsive proteins in two rice cultivars differing in N utilization efficiency. *J. Integr. OMICS*, **1** (1), 78–87.

288 Riano-Pachon, D.M., Nagel, A., Neigenfind, J., Wagner, R., Basekow, R., Weber, E., Mueller-Roeber, B., Diehl, S., and Kersten, B. (2009) GabiPD: the GABI primary database – a plant integrative "omics" database. *Nucleic Acids Res.*, **37** (Suppl. 1), D954–D959.

289 Grafahrend-Belau, E., Weise, S., Koschutzki, D., Scholz, U., Junker, B.H., and Schreiber, F. (2008) MetaCrop: a detailed database of crop plant metabolism. *Nucleic Acids Res.*, **36** (Suppl. 1), D954–D958.

290 Guo, A.Y., Chen, X., Gao, G., Zhang, H., Zhu, Q.H., Liu, X.C., Zhong, Y.F., Gu, X., He, K., and Luo, J. (2008) PlantTFDB: a comprehensive plant transcription factor database. *Nucleic Acids Res.*, **36**, D966–D969.

291 Feil, R. and Berger, F. (2007) Convergent evolution of genomic imprinting in plants and mammals. *Trends Genet.*, **23**, 192–199.

292 Mallory, A.C., Elmayan, T., and Vaucheret, H. (2008) MicroRNA maturation and action: the expanding roles of ARGONAUTEs. *Curr. Opin. Plant Biol.*, **11**, 560–566.

293 Alleman, M., Sidorenko, L., Mcginnis, K., Seshadri, V., Dorweiler, J.E., White, J., Sikkink, K., and Chandler, V.L. (2006) An RNA-dependent RNA polymerase is required for paramutation in maize. *Nature*, **442**, 295–298.

294 Brinkman, A.B. and Stunnenberg, H.G. (2009) Strategies for epigenome analysis, in *Epigenomics* (eds A.C. Ferguson-Smith, J.M. Greally, and R.A. Martienssen), Springer Science + Business Media, pp. 3–18.

295 Meissner, A. and Bernstein, B.E. (2009) Sequencing the epigenome, in *Epigenomics* (eds A.C. Ferguson-Smith, J.M. Greally, and R.A. Martienssen), Springer Science + Business Media, pp. 19–36.

296 Bernstein, B.E., Meissner, A., and Lander, E.S. (2007) The mammalian epigenome. *Cell*, **128**, 669–681.

297 Lister, R., O'Malley, R.C., Tonti-Filippini, J., Gregory, B.D., Berry, C.C., Millar, A.H., and Ecker, J.R. (2008) Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell*, **133**, 523–536.

298 Reinders, J. and Paszkowski, J. (2009) Unlocking the *Arabidopsis* epigenome. *Epigenetics*, **4**, 557–563.

299 Chinnusamy, V. and Zhu, J.K. (2009) Epigenetic regulation of stress responses in plants. *Curr. Opin. Plant Biol.*, **12**, 133–139.

300 Kim, J.M., To, T.K., Nishioka, T., and Seki, M. (2010) Chromatin regulation functions in plant abiotic stress responses. *Plant Cell Environ.*, **33** (4), 604–611.

301 Zhang, K., Sridhar, V.V., Zhu, J., Kapoor, A., and Zhu, J.K. (2007) Distinctive core histone post-translational modification patterns in *Arabidopsis thaliana*. *PLoS One*, **2** (11), e1210. doi: 10.1371/journal.pone.0001210

302 Chinnusamy, V. and Zhu, J.K. (2010) Epigenetic regulation: chromatin modeling and small RNAs, in *Abiotic Stress Adaptation in Plants: Physiological, Molecular and Genomic Foundation* (eds A. Ashwani Pareek, S.K. Sopory and H.J. Bohnert), Springer, Dordrecht, pp. 217–241.

303 Wysocka, J., Swigut, T., Xiao, H., Milne, T.A., Kwon, S.Y., Landry, J., Kauer, M., Tackett, A.J., Chait, B.T., Badenhorst, P., Wu, C., and Allis, C.D. (2006) A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature*, **442**, 86–90.

304 Kim, J.M., To, T.K., Ishida, J., Morosawa, T., Kawashima, M., Matsui, A., Toyoda, T., Kimura, H., Shinozaki, K., and Seki, M. (2008) Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in *Arabidopsis thaliana*. *Plant Cell Physiol.*, **49**, 1580–1588.

305 Kwon, C.S., Lee, D., Choi, G., and Chung, W.I. (2009) Histone occupancy-dependent and -independent removal of H3K27 trimethylation at cold-responsive genes in *Arabidopsis*. *Plant J.*, **60**, 112–121.

306 Stockinger, E.J., Mao, Y., Regier, M.K., Triezenberg, S.J., and Thomashow, M.F. (2001) Transcriptional adaptor and histone acetyltransferase proteins in *Arabidopsis* and their interactions with *CBF1*, a transcriptional activator involved in cold regulated gene expression. *Nucleic Acids Res.*, **29**, 1524–1533.

307 Scippa, G.S., Di Michele, M., Onelli, E., Patrignani, G., Chiatante, D., and Bray, E.A. (2004) The histone-like protein H1-S and the response of tomato leaves to water deficit. *J. Exp. Bot.*, **55**, 99–109.

308 Fu, W., Wu, K., and Duan, J. (2007) Sequence and expression analysis of

histone deacetylases in rice. *Biochem. Biophys. Res. Commun.*, **356**, 843–850.

309 Tsuji, H., Saika, H., Tsutsumi, N., Hirai, A., and Nakazono, M. (2006) Dynamic and reversible changes in histone H3-Lys4 methylation and H3 acetylation occurring at submergence-inducible genes in rice. *Plant Cell Physiol.*, **47**, 995–1003.

310 Pandey, R., Muller, A., Napoli, C.A., Selinger, D.A., Pikaard, C.S., Richards, E.J., Bender, J., Mount, D.W., and Jorgensen, R.A. (2002) Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis* thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Res.*, **30**, 5036–5055.

311 Papaefthimiou, D., Likotrafiti, E., Kapazoglou, A., Bladenopoulos, K., and Tsaftaris, A. (2010) Epigenetic chromatin modifiers in barley: III. Isolation and characterization of the barley GNAT-MYST family of histone acetyltransferases and responses to exogenous ABA. *Plant Physiol. Biochem.*, **48**, 98–107.

312 Demetriou, K., Kapazoglou, A., Bladenopoulos, K., and Tsaftaris, A.S. (2009) Epigenetic chromatin modifiers in barley: II. Characterization and expression analysis of the HDA1 family of barley histone deacetylases during development and in response to jasmonic acid. *Plant Mol. Biol. Rep.*, **28**, 9–21.

313 Demetriou, K., Kapazoglou, A., Tondelli, A., Francia, E., Stanca, M.A., Bladenopoulos, K., and Tsaftaris, A.S. (2009) Epigenetic chromatin modifiers in barley: I. Cloning, mapping and expression analysis of the plant specific HD2 family of histone deacetylases from barley, during seed development and after hormonal treatment. *Physiol. Plant.*, **136**, 358–368.

314 Hennig, L. and Derkacheva, M. (2009) Diversity of Polycomb group complexes in plants: same rules, different players? *Trends Genet.*, **25**, 414–423.

315 Kapazoglou, A., Tondelli, A., Papaefthimiou, D., Ampatzidou, H., Francia, E., Stanca, M.A., Bladenopoulos, K., and Tsaftaris, A.S. (2010) Epigenetic chromatin modifiers in barley: IV. The study of barley Polycomb group (PcG) genes during seed development and in response to external ABA. *BMC Plant Biol.*, **10**, 73.

316 Alexandre, C., Moller-Steinbach, Y., Schonrock, N., Gruissem, W., and Hennig, L. (2009) *Arabidopsis msi1* is required for negative regulation of the response to drought stress. *Mol. Plant*, **2**, 675–687.

317 Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature*, **416**, 556–560.

318 Cao, X. and Jacobsen, S.E. (2002) Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. *Proc. Natl. Acad. Sci. USA*, **99**, 16491–16498.

319 Radchuk, V.V., Sreenivasulu, N., Radchuk, R.I., Wobus, U., and Weschke, W. (2005) The methylation cycle and its possible functions in barley endosperm development. *Plant Mol. Biol.*, **59**, 289–307.

320 Choi, C.S. and Sano, H. (2007) Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. *Mol. Genet. Genomics*, **277**, 589–600.

321 Steward, N., Ito, M., Yamaguchi, Y., Koizumi, N., and Sano, H. (2002) Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. *J. Biol. Chem.*, **277**, 37741–37746.

322 Kovarik, A., Koukalova, B., Bezdek, M., and Opatrn, Z. (1997) Hypermethylation of tobacco heterochromatic loci in response to osmotic stress. *Theor. Appl. Genet.*, **95**, 301–306.

323 Labra, M., Ghiani, A., Citterio, S., Sgorbati, S., Sala, F., Vannini, C., Ruffini-Castiglione, M., and Bracale, M. (2002) Analysis of cytosine methylation pattern in response to water deficit in pea root tips. *Plant Biol. (Stuttg.)*, **4**, 694–699.

324 Ruan, Y., Le Ber, P., Huing, H., and Liu, E.T. (2004) Interrogating the transcriptome. *Trends Biotechnol.*, **22**, 23–30.

325 Matsui, A., Ishida, J., Morosawa, T., Mochizuki, Y., Kaminuma, E., Endo, T.A., Okamoto, M., Nambara, E., Nakajima, M., Kawashima, M., Satou, M., Kim, J.M., Kobayashi, N., Toyoda, T., Shinozaki, K., and Seki, M. (2008) *Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant Cell Physiol.*, **49**, 1135–1149.

326 Zeller, G., Henz, S.R., Widmer, C.K., Sachsenberg, T., Ratsch, G., Weigel, D., and Laubinger, S. (2009) Stress-induced changes in the *Arabidopsis* thaliana transcriptome analyzed using whole-genome tiling arrays. *Plant J.*, **58**, 1068–1082.

327 Hirayama, T. and Shinozaki, K. (2010) Research on plant abiotic stress responses in the post-genome era: past, present and future. *Plant J.*, **61**, 1041–1052.

328 Seitz, H. (2009) Redefining microRNA targets. *Curr. Biol.*, **19**, 870–873.

329 Covarrubias, A.A. and Reyes, J.L. (2010) Post-transcriptional gene regulation of salinity and drought responses by plant microRNAs. *Plant Cell Environ.*, **33**, 481–489.

330 Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R., and Zhu, J.K. (2005) Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell*, **123**, 1279–1291.

331 Zhao, B., Liang, R., Ge, L., Li, W., Xiao, H., Lin, H., Ruan, K., and Jin, Y. (2007) Identification of drought-induced microRNAs in rice. *Biochem. Biophys. Res. Commun.*, **354**, 585–590.

332 Sunkar, R., Zhou, X., Zheng, Y., Zhang, W., and Zhu, J.K. (2008) Identification of novel and candidate miRNAs in rice by high throughput sequencing. *BMC Plant Biol.*, **8**, 25.

333 Colaiacovo, M., Subacchi, A., Bagnaresi, P., Lamontanara, A., Cattivelli, L., and Faccioli, P. (2010) A computational-based update on microRNAs and their targets in barley (*Hordeum vulgare* L.). *BMC Genomics*, **11**, 595.

334 Probst, A.V., Fagard, M., Proux, F., Mourrain, P., Boutet, S., Earley, K., Lawrence, R.J., Pikaard, C.S., Murfett, J., Furner, I., Vaucheret, H., and Scheid, O.M. (2004) *Arabidopsis* histone deacetylase HDA6 is required for maintenance of transcriptional gene silencing and determines nuclear organization of rDNA repeats. *Plant Cell*, **16**, 1021–1034.

335 Aufsatz, W., Mette, M.F., van der Winden, J., Matzke, M., and Matzke, A.J. (2002) HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. *EMBO J.*, **21**, 6832–6841.

336 Hollywood, K., Brison, D.R., and Goodacre, R. (2006) Metabolomics: current technologies and future trends. *Proteomics*, **6**, 4716–4723.

337 Bino, R.J., Hall, R.D., Fiehn, O., Kopka, J., Saito, K., Draper, J., Draper, J., Nikolau, B.J., Mendes, P., Roessner-Tunali, U., Beale, M.H., Trethewey, R.N., Lange, B.M., Wurtele, E.S., and Sumner, L.W. (2004) Potential of metabolomics as a functional genomics tool. *Trends Plant Sci.*, **9**, 418–425.

338 Kopka, J., Schauer, N., Krueger, S., Birkemeyer, C., Usadel, B., Burgmuller, E., Dormann, P., Weckwerth, W., Gibon, Y., Stitt, M., Willmitzer, L., Fernie, A.R., and Steinhauser, D. (2005) GMD@CSB.DB: the Golm Metabolome Database. *Bioinformatics*, **21**, 1635–1638.

339 Matsuda, F., Yonekura-Sakakibara, K., Niida, R., Kuromori, T., Shinozaki, K., and Saito, K. (2009) MS/MS spectral tag-based annotation of non-targeted profile of plant secondary metabolites. *Plant J.*, **57**, 555–577.

340 Shulaeva, V., Cortesa, D., Miller, G., and Mittler, R. (2008) Metabolomics for plant stress response. *Physiol. Plant.*, **132**, 199–208.

341 Cook, D., Fowler, S., Fiehn, O., and Thomashow, M.F. (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature

metabolome of *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **101**, 15243–15248.

342 Kaplan, F., Kopka, J., Sung, D.Y., Zhao, W., Popp, M., Porat, R., and Guy, C.L. (2007) Transcript and metabolite profiling during cold acclimation of *Arabidopsis* reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. *Plant J.*, **50**, 967–981.

343 Morsy, M.R., Jouve, L., Hausman, J.F., Hoffmann, L., and Stewart, J.M. (2007) Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. *J. Plant Physiol.*, **164**, 157–167.

344 Sanchez, D.H., Siahpoosh, M.R., Roessner, U., Udvardi, M., and Kopka, J. (2008) Plant metabolomics reveals conserved and divergent metabolic responses to salinity. *Physiol. Plant.*, **132**, 209–219.

345 Roessner, U., Patterson, J.H., Forbes, M.G., Fincher, G.B., Langridge, P., and Bacic, A. (2006) An investigation of boron toxicity in barley using metabolomics. *Plant Physiol.*, **142**, 1087–1101.

346 Huang, C.Y., Roessner, U., Eickmeier, I., Genc, Y., Callahan, D.L., Shirley, N., Langridge, P., and Bacic, A. (2008) Metabolite profiling reveals distinct changes in carbon and nitrogen metabolism in phosphate-deficient barley plants (*Hordeum vulgare* L.). *Plant Cell Physiol.*, **49**, 691–703.

347 Maruyama, K., Takeda, M., Kidokoro, S., Yamada, K., Sakuma, Y., Urano, K., Fujita, M., Yoshiwara, K., Matsukura, S., Morishita, Y., Sasaki, R., Suzuki, H., Saito, K., Shibata, D., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2009) Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by *DREB1A* and *DREB2A*. *Plant Physiol.*, **150**, 1972–1980.

348 Urano, K., Maruyama, K., Ogata, Y., Morishita, Y., Takeda, M., Sakurai, N., Suzuki, H., Saito, K., Shibata, D., Kobayashi, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2009) Characterization of the ABA-regulated global responses to dehydration in *Arabidopsis* by metabolomics. *Plant J.*, **57**, 1065–1078.

349 Fu, J., Keurentjes, J.J., Bouwmeester, H., America, T., Verstappen, F.W., Ward, J.L., Beale, M.H., de Vos, R.C.H., Dijkstra, M., Scheltema, R.A., Johannes, F., Koornneef, M., Vreugdenhil, D., Breitling, R., and Jansen, R.C. (2009) System-wide molecular evidence for phenotypic buffering in *Arabidopsis*. *Nat. Genet.*, **41**, 166–167.

350 Morreel, K., Goeminne, G., Storme, V., Sterck, L., Ralph, J., Coppieters, W., Breyne, P., Steenackers, M., Georges, M., Messens, E., and Boerjan, W. (2006) Genetical metabolomics of flavonoid biosynthesis in *Populus*: a case study. *Plant J*, **47** 224–237.

351 Schauer, N., Semel, Y., Roessner, U., Gur, A., Balbo, I., Carrari, F., Pleban, T., Perez-Melis, A., Bruedigam, C., Kopka, J., Willmitzer, L., Zamir, D., and Fernie, A.R. (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. *Nat. Biotechnol.*, **24**, 447–454.

352 Laurentin, H., Ratzinger, A., and Karlovsky, P. (2008) Relationship between metabolic and genomic diversity in sesame (*Sesamum indicum* L). *BMC Genomics*, **9**, 250.

353 Baxter, I. (2009) Ionomics: studying the social network of mineral nutrients. *Curr. Opin. Plant Biol.*, **12**, 381–386.

354 Salt, D.E., Baxter, I., and Lahner, B. (2008) Ionomics and the study of the plant ionome. *Ann. Rev. Plant Biol.*, **59**, 709–733.

355 Baxter, I.R., Vitek, O., Lahner, B., Muthukumar, B., Borghi, M., Morrissey, J., Guerinot, M.L., and Salt, D.E. (2008) The leaf ionome as a multivariable system to detect a plant's physiological status. *Proc. Natl. Acad. Sci. USA*, **105**, 12081–12086.

356 Bentsink, L., Yuan, K., Koorneef, M., and Vreugdenhil, D. (2003) The genetics of phytate and phosphate accumulation in seeds and leaves of *Arabidopsis thaliana,* using natural variation. *Theor. Appl. Genet.*, **106**, 1234–1243.

357 Payne, K., Bowen, H., Hammond, J., Hampton, C., Lynn, J., Mead, A., Swarup, K., Bennett, M.J., White, P.J., and Broadley, M.R. (2004) Natural genetic variation in caesium (Cs) accumulation by *Arabidopsis thaliana*. *New Phytol.*, **162**, 535–548.

358 Zhang, L., Byrne, P.F., and Pilon-Smits, E.A. (2006) Mapping quantitative trait loci associated with selenate tolerance in *Arabidopsis thaliana*. *New Phytol.*, **170**, 33–42.

359 Vreugdenhil, D., Aarts, M.G.M., Koornneef, M., Nelissen, H., and Ernst, W.H.O. (2004) Natural variation and QTL analysis for cationic mineral content in seeds of *Arabidopsis thaliana*. *Plant Cell Environ.*, **27**, 828–839.

360 Loudet, O., Saliba-Colombani, V., Camilleri, C., Calenge, F., Gaudon, V., Koprivova, A., North, K.A., Kopriva, S., and Daniel-Vedele, F. (2007) Natural variation for sulfate content in *Arabidopsis thaliana* is highly controlled by *APR2*. *Nat. Genet.*, **39**, 896–900.

361 Buescher, E., Achberger, T., Amusan, I., Giannini, A., Ochsenfeld, C., Rus, A., Lahner, B., Hoekenga, O., Yakubova, E., Harper, J.F., Guerinot, M.L., Zhang, M., Salt, D.E., and Baxter, I.R. (2010) Natural genetic variation in selected populations of *Arabidopsis thaliana* is associated with ionomic differences. *PLoS One*, **5**, e11081. doi: 10.1371/journal.pone.0011081

362 Watanabe, T., Broadley, M.R., Jansen, S., White, P.J., Takada, J., Satake, K., Takamatsu, T., Tuah, S.J., and Osaki, M. (2007) Evolutionary control of leaf element composition in plants. *New Phytol.*, **174**, 516–523.

363 Kolukisaoglu, U. and Thurow, K. (2010) Future and frontiers of automated screening in plant sciences. *Plant Sci.*, **178**, 476–484.

364 Berger, B., Parent, B., and Tester, M. (2010) High-throughput shoot imaging to study drought responses. *J. Exp. Bot.*, **61**, 3519–3528.

365 Munns, R., James, R.A., Sirault, X.R.R., Furbank, R.T., and Jones, H.G. (2010) New phenotyping methods for screening wheat and barley for beneficial responses to water deficit. *J. Exp. Bot.*, **61**, 3499–3507.

366 Fedoroff, N.V., Battisti, D.S., Beachy, R.N., Cooper, P.J.M., Fischhoff, D.A., Hodges, C.N., Knauf, V.C., Lobell, D., Mazur, B.J., Molden, D., Reynolds, M.P., Ronald, P.C., Rosegrant, M.W., Sanchez, P.A., Vonshak, A., and Zhu, J.-K. (2010) Radically rethinking agriculture for the 21st century. *Science*, **327**, 833–834.

367 Street, N., Jansson, S., and Hvidsten, T.R. (2011) A systems biology model of the regulatory network in Populus leaves reveals interacting regulators and conserved regulation. *BMC Plant Biol.*, **11**, 13.

368 Nakashima, K., Fujita, Y., Kanamori, N., Katagiri, T., Umezawa, T., Kidokoro, S., Maruyama, K., Yoshida, T., Ishiyama, K., Kobayashi, M., Shinozaki, K., and Shinozaki, K.Y. (2009) Three *Arabidopsis* SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant Cell Physiol.*, **50**, 1345–1363.

369 Chen, W.J. and Zhu, T. (2004) Networks of transcription factors with roles in environmental stress response. *Trends Plant Sci.*, **12**, 591–596.

370 Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Z., Chen, X., Lam, S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J.L., Wang, X., and Zhu, T. (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell*, **14** (3), 559–574.

371 Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling

networks. *Curr. Opin. Plant Biol.*, **9**, 436–442.

372 Ma, S. and Bohnert, H.J. (2007) Integration of *Arabidopsis thaliana* stress related transcript profiles, promoter structures, and cell-specific expression. *Genome Biol.*, **8**, R49.

373 Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNA from *Arabidopsis*. *Plant Cell*, **16**, 2001–2019.

374 Urano, K., Kurihara, Y., Seki, M., and Shinozaki, K. (2010) "Omics" analyses of regulatory networks in plant abiotic stress responses. *Curr. Opin. Plant Biol.*, **13** (2), 132–138.

375 Vickers, C.E., Gershenzon, J., Lerdau, M.T., and Loreto, F. (2009) A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nat. Chem. Biol.*, **5**, 283–291.

376 Holopainen, J.K. and Gershenzon, J. (2010) Multiple stress factors and the emission of plant VOCs. *Trends Plant Sci.*, **15** (3). doi: 10.1016/j.tplants.2010.01.006

377 Dinneny, J.R., Long, T.A., Wang, J.Y., Jung, J.W., Mace, D., Pointer, S., Barron, C., Brady, S.M., Schiefelbein, J., and Benfey, P.N. (2008) Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. *Science*, **320**, 942–945.

378 Collins, N.C., Tardieu, F., and Tuberosa, R. (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? *Plant Physiol.*, **147**, 469–486.

379 Robertson, D.S. (1985) A possible technique for isolating genic DNA for quantitative traits in plants. *J. Theor. Biol.*, **117**, 1–10.

380 Noble, D. (2011) Differential and integral views of genetics in computational systems biology. *Interface Focus*, **1** (1), 7–15.

381 Yuan, J.S., Galbraith, D.W., Dai, S.Y., Griffin, P., and Stewart, C.N. (2008) Plant systems biology comes of age. *Trends Plant Sci.*, **13** (4), 165–171.

382 Libault, M., Brechenmacher, L., Cheng, J., Xu, D., and Stacey, G. (2010) Root hair systems biology. *Trends Plant Sci.*, **15** (11). doi: 10.1016/j.tplants.2010.08.010

383 Raes, J. and Bork, P. (2008) Molecular eco-systems biology: towards an understanding of community function. *Nat. Rev.*, **6**, 693–698.

384 Keurentjes, J.J.B., Angenent, G.C., Dicke, M., Dos Santos, V.A.P.M., Molenaar, J., van der Putten, W.H., de Ruiter, P.C., Struik, P.C., and Thomma, B.P.H.J. (2010) Redefining plant systems biology: from cell to ecosystem. *Trends Plant Sci.* doi: 10.1016/j.tplants.2010.12.002

385 Joosen, R.V.L., Ligterink, W., Hilhorst, H.W.M., and Keurentjes, J.J.B. (2009) Advances in genetical genomics of plants. *Curr. Genomics*, **10** (8), 540–549.

386 Kliebenstein, D.J. (2010) Systems biology uncovers the foundation of natural genetic diversity. *Plant Physiol.*, **152**, 480–486.

387 Li, Y., Breitling, R., and Jansen, R.C. (2008) Generalizing genetical genomics: getting added value from environmental perturbation. *Trends Genet.*, **24** (10), 518–524.

388 Weckwerth, W. (2008) Integration of metabolomics and proteomics in molecular plant physiology: coping with the complexity by data-dimensionality reduction. *Physiol. Plant.*, **132** (2), 176–189.

389 Weston, D.J., Gunter, L.E., Rogers, A., and Wullschleger, S.D. (2008) Connecting genes, coexpression modules, and molecular signatures to environmental stress phenotypes in plants. *BMC Syst. Biol.*, **2** (16). doi: 10.1186/1752-0509-2-16

390 Barabasi, A.L. and Oltvai, Z.N. (2004) Network biology: understanding the cell's functional organization. *Nat. Rev. Genet.*, **5**, 101–113.

391 Le Novere, N., Hucka, M., Mi, H., Moodie, S., Schreiber, F., Sorokin, A., Demir, E., Wegner, K., Aladjem, M.I., Wimalaratne, S.M., Bergman, F.T., Gauges, R., Ghaza, P., Kawaji, H., Li, L., Matsuoka, Y., Villeger, A., Boyd, S.E., Calzone, L., Courtot, M., Dogrusoz, U., Freeman, T.C., Funahashi, A., Ghosh, S., Jouraku, A., Kim, S., Kolpakov, F., Luna, A., Sahle, S., Schmidt, E., Watterson, S., Wu, G., Goryanin, I., Kell, D.B., Sander,

C., Sauro, H., Snoep, J.L., Kohn, K., and Kitano, H. (2009) The systems biology graphical notation. *Nat. Biotechnol.*, **27** 735–741.

392 Fucile, G., Di Biase, D., Nahal, H., La, G., Khodabandeh, S., Chen, Y., Easley, K., Christendat, D., Kelley, L., and Provart, N.J. (2011) ePlant and the 3D data display initiative: integrative systems biology on the World Wide Web. *PLoS One*, **6** (1), e15237.

393 Druka, A., Druka, I., Centeno, A.G., Li, H., Sun, Z., Thomas, W.T., Bonar, N., Steffenson, B.J., Ullrich, S.E., Kleinhofs, A., Wise, R.P., Close, T.J., Potokina, E., Luo, Z., Wagner, C., Schweizer, G.F., Marshall, D.F., Kearsey, M.J., Williams, R.W., and Waugh, R. (2008) Towards systems genetic analyses in barley: integration of phenotypic, expression and genotype data into GeneNetwork. *BMC Genet.*, **18** (9), 73.

394 Orr, H.A. (1998) The population genetics of adaptation: the distribution of factors fixed during adaptive evolution. *Evolution*, **52**, 935–949.

395 Farrall, M. (2004) Quantitative genetic variation: a post-modern view. *Hum. Mol. Genet.*, **13**, R1–R7.

396 Buckler, E.S., Holland, J.B., Bradbury, P.J., Acharya, C.B., Brown, P.J., Browne, C., Ersoz, E., Flint-Garcia, S., Garcia, A., Glaubitz, J.C., Goodman, M.M., Harjes, C., Guill, K., Kroon, D.E., Larsson, S., Lepak, N.K., Li, H., Mitchell, S.E., Pressoir, G., Peiffer, J.A., Rosas, M.O., Rocheford, T.R., Romay, M.C., Romero, S., Salvo, S., Sanchez Villeda, H., da Silva, H.S., Sun, Q., Tian, F., Upadyayula, N., Ware, D., Yates, H., Yu, J., Zhang, Z., Kresovich, S., and McMullen, M.D. (2009) The genetic architecture of maize flowering time. *Science*, **325** (5941), 714–718.

397 Bergelson, J. and Roux, F. (2010) Towards identifying genes underlying ecologically relevant traits in *Arabidopsis thaliana*. *Nat. Rev. Genet.*, **11**, 867–879.

398 Tardieu, F. and Tuberosa, R. (2010) Dissection and modelling of abiotic stress tolerance in plants. *Curr. Opin. Plant Biol.*, **13** (2), 206–212.

399 McLaren, A. and Michie, D. (1958) An effect of uterine environment upon skeletal morphology of the mouse. *Nature*, **181**, 1147–1148.

400 Lu, X.Y. and Huang, X.L. (2008) Plant miRNAs and abiotic stress responses. *Biochem. Biophys. Res. Commun.*, **368**, 458–462.

401 Yao, Y., Ni, Z., Peng, H., Sun, F., Xin, M., Sunkar, R., Zhu, J., and Sun, Q. (2010) Non-coding small RNAs responsive to abiotic stress in wheat (*Triticum aestivum* L.). *Funct. Integr. Genomics*, **10**, 187–190.

402 Rymarquis, L.A., Kastenmayer, J.P., Huttenhofer, A.G., and Green, P.J. (2008) Diamonds in the rough: mRNA-like non-coding RNAs. *Trends Plant Sci.*, **13** (7), 329–334.

403 De Lucia, F. and Dean, C. (2010) Long non-coding RNAs and chromatin regulation. *Curr. Opin. Plant Biol.*, **14**, 1–6.

404 Freeling, M. and Subramaniam, S. (2009) Conserved noncoding sequences (CNSs) in higher plants. *Curr. Opin. Plant Biol.*, **12** (2), 126–132.

405 Brunner, S., Fengler, K., Morgante, M., Tingey, S., and Rafalski, A. (2005) Evolution of DNA sequence nonhomologies among maize inbreds. *Plant Cell*, **17**, 343–360.

406 Salvi, S., Sponza, G., Morgante, M., Tomes, D., Niu, X., Fengler, K.A., Meeley, R., Ananiev, E.V., Svitashev, S., Bruggemann, E., Li, B., Hainey, C.F., Radovic, S., Zaina, G., Rafalski, J.A., Tingey, S.V., Miao, G.H., Phillips, R.L., and Tuberosa, R. (2007) Conserved noncoding genomic sequences associated with a flowering-time quantitative trait locus in maize. *Proc. Natl. Acad. Sci. USA*, **104** (27), 11376–11381.

407 von Korff, M., Radovic, S., Choumane, W., Stamati, K., Udupa, S.M., Grando, S., Ceccarelli, S., Mackay, I., Powell, W., Baum, M., and Morgante, M. (2009) Asymmetric allele-specific expression in relation to developmental variation and drought stress in barley hybrids. *Plant J.*, **59** (1), 14–26.

408 Zhang, X., Shiu, S.H., Cal, A., and Borevitz, J.O. (2008) Global analysis of

genetic, epigenetic and transcriptional polymorphisms in *Arabidopsis thaliana* using whole genome tiling arrays. *PLoS Genet.*, **4** (3), e1000032. doi: 10.1371/journal.pgen.1000032

409 Beckmann, J.S., Estivill, X., and Antonarakis, S.E. (2007) Copy number variants and genetic traits: closer to the resolution of phenotypic to genotypic variability. *Nat. Rev. Genet.*, **8**, 639–646.

410 Sudmant, P.H., Kitzman, J.O., Antonacci, F., Alkan, C., Malig, M., Tsalenko, A., Sampas, N., Bruhn, L., Shendure, J., Eichler, E.E., and 1000 Genomes Project (2010) Diversity of human copy number variation and multicopy genes. *Science*, **330** (6004), 641–646.

411 Estivill, X. and Armengol, L. (2007) Copy number variants and common disorders: filling the gaps and exploring complexity in genome-wide association studies. *PLoS Genet.*, **3** (10), e190.

412 DeBolt, S. (2010) Copy number variation shapes genome diversity in *Arabidopsis* over immediate family generational scales. *Genome Biol. Evol.*, **2**, 441–453.

413 Beló, A., Beatty, M.K., Hondred, D., Fengler, K.A., Li, B., and Rafalski, A. (2010) Allelic genome structural variations in maize detected by array comparative genome hybridization. *Theor. Appl. Genet.*, **120** (2), 355–67.

414 Swanson-Wagner, R.A., Eichten, S.R., Kumari, S., Tiffin, P., Stein, J.C., Ware, D., and Springer, N.M. (2010) Pervasive gene content variation and copy number variation in maize and its undomesticated progenitor. *Genome Res.*, **20**, 1689–1699.

415 Morgante, M., De Paoli, E., and Radovic, S. (2007) Transposable elements and the plant pan-genomes. *Curr. Opin. Plant Biol.*, **10**, 149–155.

416 Yin, X. and Struik, P.C. (2008) Applying modelling experiences from the past to shape crop systems biology: the need to converge crop physiology and functional genomics. *New Phytol.*, **179** (3), 629–642.

417 Li, P., Ponnala, L., Gandotra, N., Wang, L., Si, Y., Tausta, S.L., Kebrom, T.H., Provart, N., Patel, R., Myers, C.R., Reidel, E.J., Turgeon, R., Liu, P., Sun, Q., Nelson, T., and Brutnell, T.P. (2010) The developmental dynamics of the maize leaf transcriptome. *Nat. Genet.*, **42** (12), 1060–1067.

418 Setter, T.L., Conocono, E.A., Egdane, J.A., and Kropff, M.J. (1995) Possibility of increasing yield potential of rice by reducing panicle height in the canopy. I. Effects of panicle on light interception and canopy photosynthesis. *Aust. J. Plant Physiol.*, **22**, 441–451.

419 Mitchell, P.L. and Sheehy, J.E. (2006) Supercharging rice photosynthesis to increase yield. *New Phytol.*, **171** (4), 688–693.

420 Hammer, G., Cooper, M., Tardieu, F., Welch, S., Walsh, B., van Eeuwijk, F., Chapman, S., and Podlich, D. (2006) Models for navigating biological complexity in breeding improved crop plants. *Trends Plant Sci.*, **11** (12), 587–593.

421 Welch, S.M., Roe, J.L., and Dong, Z. (2003) A genetic neural network model of flowering time control in *Arabidopsis* thaliana. *Agron. J.*, **95**, 71–81.

422 Sabatini, E., Beretta, M., Sala, T., Acciarri, N., Milc, J., and Pecchioni, N. (2011) Molecular breeding. Molecular breeding, in *Tomato Genomics of Fruit and Vegetable Crops Series* (ed. J. Labate), Science Publishers Inc., Enfield, in press.

423 Friedt, W. and Ordon, F. (2007) Molecular markers for gene pyramiding and disease resistance breeding in barley, in *Genomics Assisted Crop Improvement: Vol. 2, Genomics Applications in Crops* (eds R.K. Varshney and R. Tuberosa), Springer, Dordrecht, pp. 81–101.

424 Laidò, G., Barabaschi, D., Tondelli, A., Gianinetti, A., Stanca, A.M., Li Destri Nicosia, O., Di Fonzo, N., Francia, E., and Pecchioni, N. (2009) QTL alleles from a winter feed type can improve malting quality in barley. *Plant Breed.*, **128** (6), 598–605.

425 Schmierer, D.A., Kandemir, N., Kudrna, D.A., Jones, B.L., Ullrich, S.E., and Kleinhofs, A. (2004) Molecular marker-assisted selection for enhanced yield in malting barley. *Mol. Breed.*, **14**, 463–473.

426 Toth, B., Francia, E., Rizza, F., Stanca, A.M., Galiba, G., and Pecchioni, N. (2004)

Development of PCR-based markers on chromosome 5H for assisted selection of frost-tolerant genotypes in barley. *Mol. Breed.*, **14** (3), 265–273.

427 Akar, T., Francia, E., Tondelli, A., Rizza, F., Stanca, A.M., and Pecchioni, N. (2009) Marker-assisted characterization of frost tolerance in barley (*Hordeum vulgare* L.). *Plant Breed.*, **128**, 381–386.

428 Rapacz, M., Tyrka, M., Gut, M., and Mikulski, W. (2010) Associations of PCR markers with freezing tolerance and photosynthetic acclimation to cold in winter barley. *Euphytica*, **175** (3), 293–301.

429 Neeraja, C., Maghirang-Rodriguez, R., Pamplona, A., Heuer, S., Collard, B., Septiningsih, E., Vergara, G., Sanchez, D., Xu, K., Ismail, A.M., and Mackill, D.J. (2007) A marker-assisted backcross approach for developing submergence-tolerant rice cultivars. *Theor. Appl. Genet.*, **115**, 767–776.

430 http://www.generationcp.org/sp5/?da=09148937 (8 February 2011).

431 Varshney, R.K., Graner, A., and Sorrells, M.E. (2005) Genomics-assisted breeding for crop improvement. *Trends Plant Sci.*, **10** (12), 621–630.

432 Peleman, J.D. and van der Voort, J.R. (2003) Breeding by design. *Trends Plant Sci.*, **8**, 330–334.

433 Kazmann, E. and Muller, R. (2010) The future of cereal/wheat breeding in Europe. ESA-SCP, Brussels, 11.10.2010.

35
Sugarcane: Physiological and Molecular Approaches for Improving Abiotic Stress Tolerance and Sustaining Crop Productivity

Ashok K. Shrivastava and Sangeeta Srivastava

Sugarcane, an important cash crop, due to its long duration, faces vagaries of all the seasons, and it is rather impracticable to provide favorable conditions for all the *critical stages*, spaced far apart temporally. Furthermore, overexploitation of natural resources and human activities have made our ecosphere prone to abiotic stresses that affect growth and development, chemical composition, and sugar synthesis and its accumulation in sugarcane, and ultimately affect sugarcane/sugar productivity. They also aggravate certain other abiotic and biotic stresses and affect the availability of seed cane. To defend the technological gains attained so far in sugarcane and sugar productivity, and to augment them further to face challenges of abiotic stresses physiological, breeding, and molecular approaches are important.

Among physiological approaches, criteria for selection of varieties (or parents for use in breeding programs) for tolerance to abiotic stresses are important. For management of abiotic stresses, physiological principles such as inducing hardiness, increasing the age of crop at the advent of stress, training roots to go vertically deeper in the soil, reducing the heat load and preventing further water loss from the soil, the use of moisture absorbers in the soil (which make it available during drought), and nutrient management are important in managing drought-affected and rain-fed canes. Increasing the age of the crop at the advent of waterlogging/flood (so that it suffers relatively lesser damage), planting *rayungans* or pre-germinated setts, preventing water from entering root zone, improving waterlogged soils by incorporating organic bulk manures, and using certain nutrients impart tolerance to waterlogged conditions. Control of rhizospheric salinity, management of waterlogging, adjustment of the ridge direction, the use of pregerminated setts and the use of trash veins system of planting, the use of certain nutrients can effectively manage salt-induced stress. For low-temperature tolerance, for reducing post-frost losses, altering planting time, frequent postmonsoon irrigation, and use of nutrients such as Si are important. For improving sprouting of stubble buds and productivity of winter-initiated ratoons, avoidance of low temperature for the exposed stubble (by covering with trash, polyethylene, and soil), and certain biochemical interventions using plant growth-regulating substances and nutrients appear promising. Nutrient deficiencies developed under rain-fed and saline–alkaline conditions can be effectively managed

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by application of appropriate nutrient and organic matter in the soil. Silicates are useful in overcoming the effects of low temperature and toxicity of Al and Mn in the acidic soils.

Some *Saccharum* species and related genera possessing tolerance to abiotic stresses have been identified. Their use in breeding programs may lead to development of high yield, high sugar varieties tolerant to abiotic stresses. As in nature, more than one type of stresses often occur together or the prevalence of one stress may accentuate or aggravate the other, which further increases the losses, varieties possessing *tolerance to multiple stresses* will be desirable.

Some progress has also been made in molecular interventions for inducing tolerance to abiotic stresses in sugarcane. Genes and gene products induced during hypoxia/anoxia have been identified. Real-time RT-PCR profiling of EST clusters has helped in identification of several stress clusters showing higher expression levels under water deficit stress. Accumulation of osmolytes trehalose and proline also contributes to drought tolerance. Expression of heat shock proteins and dehydrins has a definitive role under high-temperature stress. Cold-inducible ESTs in sugarcane have shown induction of novel cold-responsive genes. There is a need to understand the mechanism of action of these stress-responsive genes as to how they help sugarcane to protect itself from various stresses.

35.1 Introduction

Being a long-duration crop, sugarcane faces vagaries of all the seasons. It is rather impracticable to provide favorable conditions for all the *critical stages*, spaced far apart temporally, so as to realize its optimal production potential. Furthermore, overexploitation of natural resources and human activities have made our ecosphere prone to abiotic stresses such as drought (and shortage of irrigation water), flooding/waterlogging, salinity, high and low temperatures, soil-related problems, emerging nutrient deficiencies, and pollution. Cyclone and winds, especially in coastal areas, also affect sugarcane productivity. The use of high-yield varieties, high intensity of irrigation, and fertilizer consumption, especially the lopsided use of nitrogen and pesticides in high production-intensive agriculture, has aggravated some of these problems [1]. Abiotic stresses affect growth and development, chemical composition, sugar synthesis, and its accumulation in sugarcane, and ultimately affect sugarcane/sugar productivity. They also aggravate certain other abiotic and biotic stresses and also affect the availability of seed cane (Figure 35.1). The impact of abiotic stresses may aggravate further due to global climate change. The IPCC has projected that global mean annual surface air temperatures by the end of this century is likely to increase in the range of 1.8–4.0 C; and it is very likely that hot extremes, heat waves, and heavy precipitation event may become more frequent in times to come [2].

Sugarcane requires large amount of water, but it cannot withstand "wet feet" and has certain temperature optima for various growth processes and stages of devel-

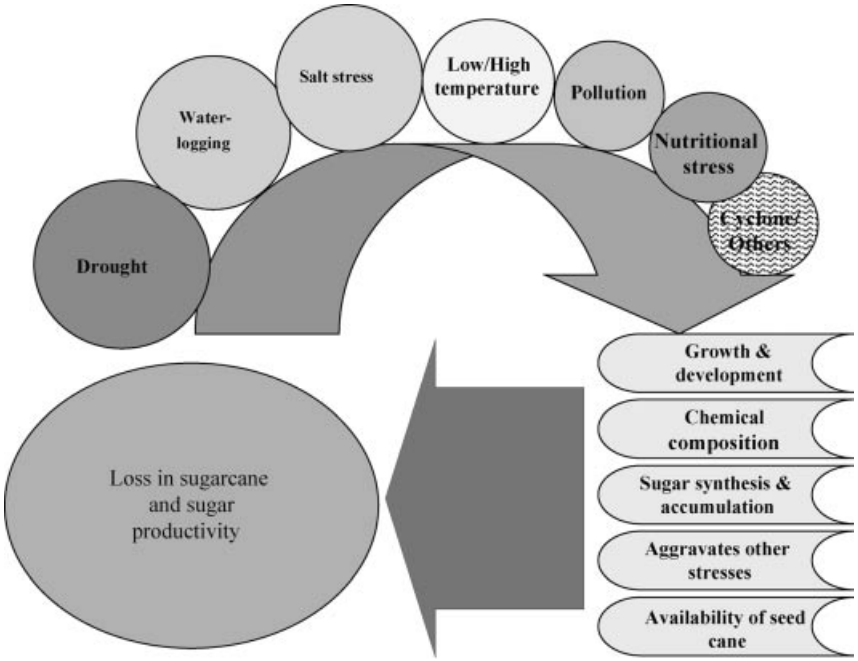

Figure 35.1 Abiotic stresses affecting sugarcane and sugarcane productivity (reproduced from Ref. [1]).

opment. Poor natural ripening conditions such as high temperature and excessive rainfall/soil moisture exist in most of the sugarcane growing areas of the world. In India, rain-fed agriculture mainly exists on marginal and submarginal lands, which suffer acute soil moisture stress, and is often accompanied by multinutrient (K and Zn) deficiencies. Low temperatures prevailing at harvest, especially in subtropical India, affect sprouting of stubble buds *vis-à-vis* productivity of the succeeding ratoon crop. Certain abiotic stresses are known to trigger off other abiotic/biotic stresses, and their cumulative impact further aggravates cane and sugar losses. Abiotic stress (es) leading to other abiotic stress(es) are as follows: waterlogging to salinity, alkalinity, and in some cases acidity, and Fe toxicity; drought to salinity; low temperatures to drought; salinity to B toxicity; high temperatures aggravate drought effects; and soil compaction leads to depressed uptake of nutrients. Abiotic stress also leads to some biotic stresses as follows: waterlogging to red rot, wilt, pineapple disease, stubble rot, accentuates white fly (especially, in N-deficient soils), scale insect, Gurdaspur borer, cut worm, and so on; drought accentuates damage due to pineapple disease and leaf scald; prolonged drought in acid soils (supplied with higher N) favor wilt development; heavy buildup of termites, shoot borer, pyrilla, mealy bugs, whiteflies, scale insects, mites, wooly aphids, and so on; salinity to salt blight, shoot borer; low temperatures to stem borer; nitrogen deficiency aggravates

white fly infestation and soil compaction leads to development of the early shoot borer [1].

Many instances in the past have been recorded when advent of abiotic stresses affected sugarcane and sugar production [1]. Recently, Central-south Brazil experienced severe drought in May–June 2010, which has resulted in decrease in sugarcane productivity by 9.1% and loss in sugarcane production to the tune of 18.7 million tons. Australia has also experienced "wetter-than-average" conditions that have both reduced sugar content and recovery [3]. In India, approximately 2.97 lakh ha of cane area is prone to drought, affecting the crop at one or the other stage of growth. Floods and waterlogging are serious problems in about 2.13 lakh ha in Eastern Uttar Pradesh, Bihar, Orissa, coastal Andhra Pradesh, and Kolhapur area of Maharashtra. Sugarcane is cultivated in about 7–8 lakh ha under saline (both in irrigated soils and in semiarid areas) conditions. Though the crop is moderately tolerant to salinity, the losses are significant [4]. Approximately, 16.9 lakh ha cane acreage (in Punjab, Haryana, and Western Uttar Pradesh) is prone to low temperatures; in nearly half this area (8.5 lakh ha), sprouting of the winter-initiated ratoons is affected and some areas also get occasional frost, affecting sugarcane productivity.

35.2
Physiological Approaches to Improve Tolerance of Sugarcane to Abiotic Stresses

The several tillers attached to a sugarcane plant (and leaves and roots) make a *physiologic compensatory continuum* that imparts the sugarcane plant an ability to tide over many abiotic stress conditions, gaps in the field, varying nutrient levels, infestation of pests, and so on [5]. Sugarcane has enormous compensatory ability to restitute the losses incurred due to waterlogging after such conditions are over as indicated by development of normal roots [6], increased rate of tiller elongation [7], and buildup of stalk dry weight [8]. Among physiological interventions, two types of approaches have been used: developing criteria for selection/identification of varieties possessing tolerance to abiotic stress conditions and imparting tolerance to a commercial variety to a particular abiotic stress.

35.2.1
Criteria for Selection of Varieties Possessing Tolerance to Abiotic Stress

Some of the physiological characteristics, which are related to the tolerance to a particular abiotic stress, could be effectively utilized for evaluation of clones/varieties so as to improve cane and sugar productivity under such conditions. These could also be beneficial in evaluation of progeny of a cross or even a biotechnological transformation through which transfer of a trait of economic importance is being attempted.

35.2.1.1 Drought Tolerance

- Thin-stalked varieties with more number of millable canes [9].
- Lower shoot:root ratio [10].
- Maintaining higher leaf sheath moisture (<76%) [11].
- Accumulation of free proline under water stress [12, 13].
- Release of more drought-induced electrolytes [14].

35.2.1.2 Waterlogging Tolerance

- Higher extension growth [1].
- Screening under simulated waterlogged conditions [15, 16].
- Sugarcane varieties (namely, Co 951, Co 975, and Co 62175) that retain higher stalk population and maintain top foliage green during flooding and after recession of floodwaters perform well under waterlogged conditions [17].
- Higher phenol oxidase activity [18].
- Less increase in alcohol dehydrogenase (ADH) activity during anoxia [19].

35.2.1.3 Tolerance to Salt-Induced Stress

- Pink pigmentation and waxiness in varieties [20].
- Selection in tissue culture: 0–5% differentiated callus of sugarcane is transferred to Murashige and Skoog medium containing 20% coconut water and 18–20 g l^{-1} NaCl. Survivors were isolated after 5 months. These were grown for additional 5–13 months in a medium containing 10–20 g NaCl l^{-1} and the resultant tolerant plants were field tested and evaluated for salt resistance [21].
- Single-bud setts of nearly uniform size are placed in jars containing 200 ml of NaCl solution (0, 2, 4, and 6 atmospheres of osmotic pressure). The sett is so placed that the bud remains just above the solution level. These solutions were changed every third day. On the 30th day, the setts were removed and shoot weight was recorded. The variety in which there was relatively minimum decline in shoot weight (as in the case of Co 449) was rated as salinity tolerant [22].
- At Central Soil Salinity Research Institute, Karnal (India), sugarcane germplasm is evaluated for tolerance to salinity–alkalinity at an ESP of 45 (pH 9.5) [23]. Evaluation of sugarcane varieties under sodic and saline conditions has also been done by observing performance in response to natural variation in ESP and EC in Australia [24] and in EC in Colombia [25].
- Ability to restrain Cl^- uptake and its accumulation in leaf laminae [26].

35.2.1.4 Tolerance to Low-Temperature Stress

- Proportion of leaf tissue that remained green after exposure to low temperatures [27].
- Bud mortality index, plant mortality index, and field performance [28]. It is a more comprehensive system.

- Subjecting setts/settlings to low temperatures: 3–5 °C for 9 h (setts) and 3–5 °C for 5 days (6–8 week-old settlings and change in osmotic concentration of sap intenseness was used to distinguish tolerant and susceptible varieties [29]. Freezing treatment (-2.5 ± 0.2 °C) for 6 h for 2 days in a deep freeze at 4–6 leaf stage of the settlings appeared to be desirable.
- Mannitol and isomaltotriose content in cane juice [30].
- Presence of certain peroxidase enzyme bands [31].
- Increase in lactic acid [32].
- Electrolyte leakage [33].

35.2.1.5 Tolerance to High Temperatures

- Subjecting samples of $+1$ leaf to high temperature (55 °C) in a water bath for 15 min and leaf tissue damage was assessed. Varieties least sensitive (<30% damage) were identified as tolerant to such conditions [34].
- First the leaf tissues is acclimated to heat and then exposed either to various temperatures for a set period of time (heat killing temperature) or to a given high temperature for various periods of time (heat killing time) and death of tissues is noted by electrolyte leakage by observing alterations in electrical conductivity. Tolerance is usually estimated as time or temperature required to kill 50% of the tissues [35].

Varieties/clones selected so could be used as tolerant variety in areas prone to a particular stress or may be used in breeding to impart the tolerance trait to particular abiotic stress.

35.2.2 Imparting Tolerance to a Commercial Variety to a Particular Abiotic Stress

35.2.2.1 For Drought Conditions

Drought is the most important water-related problem of sugarcane agriculture worldwide. It is caused by annual precipitation being less than the normal, late onset of monsoon, early withdrawal of monsoon, long intervening dry spell in the monsoon period, and declining groundwater levels at faster rate. Low and high temperatures and salinity and alkalinity also accentuate it. Types of drought experienced by a sugarcane crop [1] are as follows:

i) Occasional drought.
ii) Frequent drought: shortage of water and prolonged drought conditions as in Maharashtra and Telangana in Andhra Pradesh in India, Sindh Province in Pakistan, southern cane growing belt in China, and so on.
iii) Rain-fed conditions: characterized by frequent drought spells (leading to severe moisture stress in soil–plant system) and multinutrient (K and Zn) deficiencies.
iv) Experienced by the crop during dry spells in its growth cycle (available moisture is not sufficient enough to maintain growth); for example, April–June in

subtropical India, October–April in Lao PDR, Guatemala, June–August in Japan, June–September in Papua New Guinea, and so on.

Any initial setback received by the crop during summer for want of irrigation hampers tillering and the plant cannot compensate it later if the subsequent growth span is short. If it is experienced during ripening, overstands, as in subtropical India, cane yields decline and juice quality deteriorates. Magnitudes of losses increase if it is a flowered crop. It also affects the performance of succeeding ratoon crop. For areas prone to drought, following approaches have been adopted:

- **Inducing drought hardiness**: Soaking sugarcane setts in saturated lime solution (80 kg lime/1000 l) for 2 h a day before planting induced drought hardiness and improved cane yield. This is also helpful in areas with limited irrigation [36]. A sugarcane crop can also be hardened by withholding water for 30 days at 90 days after planting. As a result, the crop suffers less damage when it is required to face the actual moisture stress during hot summer months [37].
- **Increase the age of the crop at the advent of drought (so that it suffers less damage) – Early planting**: A sugarcane crop of relatively higher age, at the advent of drought (when the summer sets in) withstands drought better than a younger crop. In Andhra Pradesh, it is desirable to plant a 12 month crop in January to mitigate adverse effect of drought (in summer). The best time for planting an *adsali* crop is August/September for withstanding drought in the next summer [38].
- **Training the roots to go vertically deep (rather than superficially and laterally) in the soil**: Before the onset of drought, the roots must be trained to go vertically deep, rather than spread superficially and laterally so that they may obtain water from deeper layers in the soil. For such purposes, planting in deep trenches is desirable. In such trenches, soil moisture retention is improved by adding high dose of bulky manure like farm yard manure (FYM), compost, and so on before planting. In addition, trash must be spread to prevent surface evaporation of moisture. Deep trench method of planting resulted in higher cane yields at Vedapathimangalam, Tamil Nadu, where the crop experiences severe drought during formative phase [39]. Experience in subtropical India indicates that after planting in spring, it takes 40–45 days to complete germination and the crop is normally irrigated immediately after this. Delaying this first irrigation, within reasonable limits, also trains roots to go relatively deeper and impart some degree of tolerance to drought in the ensuing summer season (R.S. Verma, personal communication).
- **Nutrients imparting tolerance to drought**: A fertilized crop of sugarcane was relatively less prone to losses due to drought. In Argentina, during the drought of 1995, cane yield losses ranged from 10 to 45% (in plot fertilized with N at the rate of 100 kg ha^{-1}) compared to 12–60% in the plots raised without N fertilizer [40]. It has been observed that plants grown in a soil with K are less sensitive to wide variations in water supply [41] and the K requirement of plants centers around its four physiobiochemical roles: enzyme activation, anion neutralization, membrane transport, and maintenance of osmotic potential [42]. Its application also increased accumulation of free proline in sugarcane [43], which is an

indication of drought resistance. Sato *et al*. [44] noticed that P supply alleviates the negative effects of water deficit on sugarcane photosynthesis, possibly through the enhancement of proline content.

Under drought conditions, planting setts soaked in saturated limewater for 2 h induced drought hardiness and improved cane yield. In tropical India, under drought conditions, spraying urea + KCl (2.5% each) at 60, 90, and 120 days after planting (d.a.p.) improved cane yield [39]. Spraying of 2.5% K_2SO_4 during summer mitigated drought and improved cane yield by 10.7% [45]. Potassium application (60 kg K_2O ha^{-1}) + 2 irrigation in February and March before the onset of drought improved cane yield and juice quality [46]. At Coimbatore, where drought occurs during early stage of crop growth, application of potassium either in the soil (168 kg K_2O ha^{-1}) or its soil application (112 kg K_2O ha^{-1}) + foliar application (2.5% KCl spray at 45, 75, and 105 d.a.p.) improved both juice quality and sugar yield [47].

Si deficiency increased the rate of transpiration by 30% [48] and improved Si nutrition reduced the rate of transpiration [49], and application of Si could be helpful in imparting drought tolerance.

In rain-fed areas, which experience frequent drought spells, severe moisture stress results in soil–plant system. Besides the amount and distribution of rainfall, soil type and its depth affect the moisture availability for crop growth. Multinutrient deficiency (Zn and K) is also common in such areas. An optimal nutrient supply (N, P, K and Zn) ensured relatively deeper root system and helped in better adoption of crop *vis-à-vis* sustained productivity under such conditions [50]. Studies on other crops have shown that in rain-fed areas, under limited moisture conditions, application of phosphorus also improved crop productivity [51].

- **Reduce the heat load and prevent further water loss from the soil**: Application of trash mulch soon after emergence was very effective in reducing water evaporation from the soil and improved cane yields. Trash mulching during initiation of a ratoon crop leads to conservation of soil moisture and improved cane yield. The use of trash mulching in cane improved yield under drought-affected conditions in tropical India [45, 52–56]. In Australia, the use of green cane trash blanket improved yields under dry conditions. This was also associated with better moisture retention, no soil erosion, and better weed control [57].
- **Use of moisture absorbers in the soil that make it available during drought**: Application of organic amendments in the soil of FYM, press mud, coir waste, *etc*., ensures availability of some moisture during drought and improves cane yield under such conditions [54, 58]. Needless to mention that such amendments also improve soil physical conditions that enable desirable root spread and nutrient uptake. Besides organic amendments, the use of certain moisture absorbents (*Jalshakti*, *Stocksorb*, and *Alcosorb*), which absorb water many times of their weight, make it available subsequently to overcome drought [52, 58, 59].

- **Use of certain growth regulating substances**: Foliar spray of low concentration of ethrel (100 ppm) before likely onset of drought or soaking seed canes in it improved drought tolerance and reduced the loss of cane and sugar yield [60].

35.2.2.2 For Waterlogged Conditions

Under Indian conditions, following types of waterlogging are normally met with:

i) Soils waterlogged in rainy season (July–September in many sugarcane growing areas) at grand growth phase.
ii) Waterlogging due to high water table because of lateral seepage of water of river Ghagra (in Eastern Uttar Pradesh) affects all stages of growth.
iii) Water standing from May–July and soils saturated up to September (in Assam) affect late tillering stage and grand growth phase.
iv) Sugarcane growing in valleys and banks (*diara* lands) where crop is subjected to standing water 0.5–1.0m deep during July–September in north Bihar, Eastern Uttar Pradesh, and West Bengal.
v) Heavy rainfall accompanied by cyclonic winds during December/January in southeast coastal areas of Andhra Pradesh and Tamil Nadu (uprooting and lodging during ripening adversely affect cane quality).

If the crop is flooded with water standing more than 0.5 m deep or so for a longer time, nothing can be done except to plant waterlogging/flood-tolerant varieties, in areas frequently affected. For other types of waterlogging and stagnation of water (of less than 1 m), following approaches could be helpful:

- **Increase the age of the crop at the advent of waterlogging/flood (so that it suffers relatively lesser damage) – Early planting**: A sugarcane crop of relatively higher age, at the advent of waterlogging/flood will be damaged relatively lesser. In Kerala, a typical *eksali* zone and in Andhra Pradesh, in the areas liable to waterlogging, sugarcane is planted early on in the year so that the crop is sufficiently grown up by the time floods come in July–August [38, 61]. In Nayagarh (Orissa) where water remains standing for 30–40 cm from mid-July to end of September, in a study sugarcane was planted at monthly intervals on 15th day of each month from November to April. Cane and sugar yields were highest with cane planted on 15th November and it decreased with subsequent plantings [62].
- **Planting rayungans or pre-germinated setts**: In Kolhapur of Maharashtra, flooding during the establishment (or germination) is potentially devastating. By planting *rayungans* or pre-germinated setts, sugarcane productivity may be sustained to some extent [63].
- **Prevent water from entering root zone – Planting on ridges**: Waterlogged areas that tend to get inundated with 60–100 cm deep water, planting on ridges (in February/March) is beneficial. These are 45–60 cm high from soil surface and 90–120 cm from the bottom of the furrow in between. These furrows contain water and sugarcane remains much above it. This practice allows improvement in drainage and at the same time ensures some aeration for the roots as the root zone lies above water level. Such a planting method has sustained sugarcane produc-

tivity in waterlogged soils in Louisiana (USA) [64]. This method has also shown promise in studies conducted at Captainganj (Deoria in Eastern Uttar Pradesh), Marhowrah (Chapra), Barachakia (Motihari), and Chanpatia (Champaran) in Bihar, using sugarcane varieties CoS 8436 (early ripening), CoS 767 (mid-late ripening), and BO 91 (late ripening) [65]. In North Bihar, planting on raised seedbeds has given best results in waterlogged areas. Double-row planting along with raised beds has also improved cane yield [65].

- **Nutrients imparting tolerance to waterlogged conditions**: Under flooding or submergence, O_2 deficit in the rhizosphere results in decreased active ion uptake and nutrient deficiency develops. It has been seen that an increase in P content in the stalk was related to tolerance to flooding [66]. Application of 2% of N, under trench planting system, improved yields under such conditions. To sustain productivity of a ratoon crop, under such conditions 130 kg P_2O_5 along with 250 kg N ha^{-1} was beneficial [46].
- **Improvement of waterlogged soils by incorporating bulk organic manures**: Waterlogged soils could also be effectively managed by incorporating organic matter through FYM, compost, and green manuring in Saurashtra region of Gujarat. Application of press mud, coir waste, and groundnut shells are also beneficial under such conditions [67]. In another study, application of 3 ton ha^{-1} of press mud alone increased cane yield by 48% over waterlogged control [68].
- **Improving drainage**: In Gujarat, installing a subsurface drainage system, in a sugarcane area where water table normally fluctuated between 0 and125 cm during the year and remained near the ground surface during monsoon months improved productivity. The drainage system comprised of slotted PVC pipes laid with gravel envelopes installed at 20, 30, and 40 m drain spacing at a depth of 1 m. The improvement in sugarcane productivity was relatively more in closely spaced drains. The closer drain spacing reduced the period of water stagnation [69]. In Bichpuri Minor Command area of Uttar Pradesh, waterlogging and prolonged wetness are major crop production constraints. Here also to improve drainage, a network of surface drains and inverted siphons were constructed. This intervention led to 22% improvement in sugarcane productivity, besides an improvement in trafficking and workability in the standing crop [70]. In Haryana, proper and low frequency of irrigation protects land from high water table and secondary salinization [71]. In many coastal areas and valley soils in Puerto Rico, where internal drainage is a problem, deep tillage and installing subsurface drainage improved sugar yield [64].

35.2.2.3 For Salt-Induced Stress Conditions

In India, salt accumulation in the soils is one of the main threats to the sustenance of irrigated agriculture. Soil salinity associated with waterlogging is a common menace in all the major irrigation projects in the country. Although sugarcane has been claimed to be a highly salt-tolerant plant [72, 73], salinity, alkalinity, and acidity are important constraints to its productivity in many parts of the world.

Following approaches have been adopted to minimize yield reductions in sugarcane due to saline–alkaline conditions:

- **Control of rhizospheric salinity**: Reclamation of salt-affected soils is a two-step process involving displacing the Na held on soil exchange complex by application of a chemical amendment, such as gypsum (1.7 ton ha^{-1} to remove 1 meq Na^+ / 100 g soil), and removing this displaced sodium from the root zone by leaching with irrigation water. This may reclaim saline/alkaline soils and sustain their productivity. Gypsum requirement varies with soil type and pH [74]. In Andhra Pradesh, application of 3 ton ha^{-1} gypsum improved soil characteristics; however, 50% gypsum (1.5 ton ha^{-1}) in combination with rice husk followed by flushing with good-quality water gave the best results [75].

In Australia, on sodic soils, application of gypsum at 2 ton ha^{-1} with irrigation water improved cane yield by 20% in some of the sodic soils. Subsequent studies indicated that surface-applied gypsum (10 ton ha^{-1}) and dissolved gypsum (2 ton ha^{-1}) applied through irrigation water produced an additional 3.5 and 2.6 ton cane ha^{-1}, respectively [76]. Cane yields, in fully irrigated sodic soils (ESP < 25), were improved by up to 25% with application of gypsum at 10 ton ha^{-1} or 2 ton ha^{-1} annually dissolved in irrigation water. Reclamation of irrigated soil was also achieved by reducing the natural slope from 0.49 to 0.07% while maintaining the integrity of the topsoil [77].

In South Africa (Nkwaleni Valley), for reclaiming saline-sodic soils, 31 ton of gypsum per hectare and 6 ton of sulfur per hectare was used. In this trial, average yields of plant plus ratoon crops were 82 ton ha^{-1} (control), 99 ton ha^{-1} (addition of sulfur), and 100 ton ha^{-1} (addition of gypsum) [78]. In the saline soils of Clarendon Sugar Company, Morelands, Jamaica, application of S (330 kg ha^{-1}) reduced salinity [79].

Studies conducted in Taiwan indicated that approximately 65% of the salts (from a poorly drained and saline-sodic soil at Aoku) are removed from the soil to a depth of 100 cm by using 1000 mm of leaching water [80]. Reclaiming salt-affected soils by open ditch drainage was relatively more effective than the subsurface drainage [81]. Some models have also been developed to predict the amount of water needed to ameliorate and remove salt from a fine textured saline-sodic soil on which sugarcane was grown at Sanku farm of Chiali Sugar Mill in Taiwan [82].

Addition of molasses up to 48 qtl ha^{-1} can also be used to partially reclaim alkaline soils after which sugarcane may be planted [83].

Incorporation of bulk manures such as farm yard manure (25–40 ton ha^{-1}), press mud cake (5–7.5 ton ha^{-1}), compost (15–20 ton ha^{-1}), and green manure (using *Sesbania aculeata*) has enhanced the productivity of saline soils in Gujarat [84], Karnataka [85], Maharashtra [86], and Haryana [71]. In areas irrigated with saline water, trench planting and application of 25 ton ha^{-1} of FYM or press mud combined with trash mulching increased cane and sugar yields [87]. In Australia, at Favero Cane farm in Colevale, in salt-affected land (where cane yield was 40 ton ha^{-1}), application of 60–65 ton ha^{-1} of filter press mud increased the cane yield to 155–160 ton ha^{-1} in plant cane and 110 ton ha^{-1} in the succeeding ratoon crop. At another location,

Pioneer Mill area, the improvement was only by 20 ton ha^{-1}. Here although salinity remained twice as high as in the original soil causing severe inhibition of growth, after this treatment, soil retained more moisture and was friable rather than hard setting [88].

- **Managing waterlogging**: As mentioned earlier, waterlogging has led to salinity problem in many parts of Karnataka, Orissa, Haryana, and Punjab in India and in some other countries such as Egypt. Proper and low frequency of irrigation may protect the lands from buildup of high water table and secondary salinization. Besides, management practices for waterlogged areas should be adopted to prevent the spread of secondary salinization.
- **Adjustment of the ridge direction**: Maintaining ridge direction from east-to-west, in subtropical India where such problem exists, permits accumulation of salt on the top of the ridges and sugarcane setts may be planted on their slope [63].
- **Crop rotations**: Crop rotations provide a cultural means to keep the effects of salinity/alkalinity minimum and at the same time sustain productivity. In Maharashtra, crop rotations involving *dhaincha*–cane–cotton–onion and *adsali* cane–onion–cotton were promising [89]. Patil and Ghonsikar [86] have suggested sugarcane–cotton–rice–*Sesbania aculeata* as a desirable cropping rotation on partially reclaimed saline soils.
- **Use of pregerminated setts and use of trash-veins system of planting**: Using pregerminated setts has been recommended for saline–alkaline soils [63]. In alkaline soils, yields of sugarcane could be doubled by planting cane in deep (20–25 cm) furrows and packing trash in the furrows as soon as the germination is over and tillering starts [90].
- **Nutrient management**: Salt tolerance of crops varies with soil fertility. Experimental data support the view that standard fertilizer recommendations for nonsaline–alkaline conditions are also suitable for saline conditions [91]. Studies conducted in India indicated that N, P, and K fertilization to sugarcane on saline soils is usually observed to suppress the availability of toxic elements; K depressed Na, P, Mg, and Mo; and nitrogen increased K availability [92]. Application of additional P may increase plant growth under saline conditions [93]; however, high concentrations, which may be beneficial under nonsaline conditions, could cause injury to the plants [94]. It has been shown that high phosphorus supply, with salts, does not contribute to osmotic adjustment and instead accumulates in the cell walls [95].

In Hawaii, on some calcareous saline soils (Mamola and Molokai soils), application of DTPA Fe increased cane yields by 37.5% [96].

In sugarcane growing on saline–alkaline soils, deficiency of P, Fe, and Zn was a common occurrence. To overcome this Zende and Hapase [89] suggested the following measures:

- Enriched compost with super phosphate (2 kg P_2O_5 10 ton^{-1} compost) should be mixed in the soil before planting.
- 10–20 kg ha^{-1} of $FeSO_4$ should be added to soil before planting to counteract chlorosis. It may be sprayed three–four times if chlorosis is detected on growing crops (4 kg $FeSO_4$ 1000 l^{-1} water ha^{-1}). Iron chelates may be used.

- 10 kg ha^{-1} of $ZnSO_4$ should be mixed in the soil before planting. On the standing crop, it may be sprayed along with 4 kg ha^{-1} of $FeSO_4$ (two–three sprays at 8–10 days interval).
- Use ammonium sulfate as a source of N or add 2–5 kg sulfur with each bag of urea.
- Never allow the field to remain fallow particularly the reclaimed one. Even growing of certain grasses is beneficial.

K and Si enhanced salt tolerance in salt-sensitive sugarcane genotypes (CPF 243 and SPF 213) resulting in decreased Na^+ concentration and increased K^+ with an improvement in K^+/Na^+ ratio [97].

- **Acidic soils**: Acidity is a problem in sugarcane soils in Kenya, South Africa, Taiwan, and so on. In Kenya, a pH of 4.3–5.0 in low-lying areas and 6.0 in high lands is observed. In South Africa, most of the sugarcane soils tend to be acidic (pH 4.6–6.0) and suffer from internal drainage [64]. In India, acidic soils (with pH < 6.0) are found in Kerala, coastal Karnataka, Goa, parts of Orissa, Assam, submountainous Himalayan region, the *terai* of Uttar Pradesh and Bihar, and some parts of Madhya Pradesh and Andhra Pradesh. Crops growing on acidic soils suffer due to toxic effects of excessive Al, Fe, and Mn, high phosphate fixation, low rate of nitrification, low water holding capacity, and deficiency of Mo, Si, S, K, Ca, B, and Mg [63].

For acidic soils below pH 5.5, liming (1.2–2.0 ton ha^{-1} of lime such as CaO) is essential to correct nutrient imbalance. Instead of adding full dose at one time, 10–25% of lime requirement is added each year. Lime should be incorporated during fallow period or prior to monsoon and after this, 3–4 months time is allowed for reaction with the soil. In acidic soils of Kerala, variety Co 997 did not respond to lime applications with regard to yield, whereas yield of Co 785 increased with lime application in the first and second ratoons. In plant crop, however, yield declined sharply when 150 kg ha^{-1} of CaO was applied [98]. Studies conducted in the United States also indicated that liming acidic soils with dolomite (4.4 ton ha^{-1}) improved sugarcane productivity, and the relative improvement was rather more in a ratoon crop [99]. In Assam, in acidic soils with pH 5.5, variety Co 997 was more successful [63]. In Kenya, addition of gypsum ameliorated acidic soil and improved yields in plant and ratoon crops. At some of the locations such as Masaka, relative increase in yield was more in ratoons. In South Africa, application of 5 ton of lime + 5 ton of gypsum per hectare in acidic soil in the Kwazulu-Natal midlands improved sugarcane productivity [100].

In acidic soils (Ultisols) in Sumatra, application of phosphorus improved cane yield [101]. Application of silicon also alleviated Al and Mn toxicities [1].

35.2.2.4 Low Temperature

Effects of low temperature are bifold: (a) effects on physical, chemical, and biological properties of soil that indirectly influence growth and development (especially of the roots) and (b) direct effect on growth and development. In subtropical India, the problem of low temperature is twofold: effect of low temperature during ripening and

poor sprouting of stubble buds in a winter harvested crop leading to poor regrowth of succeeding ratoon crop and thus poor ratoon productivity [1]. Following approaches have been adopted to minimize yield reductions in sugarcane due to low-temperature conditions.

35.2.2.4.1 For Reducing Post-frost Losses

- **Preservation of seed cane and improving germination**: At the first sight of frost, canes were cut and buried in the field. The practice, however, was not beneficial as the lot of bud sprouts got damaged during handling for planting and due to termite attack. Clamping was also done to preserve seed cane, but the buds sprouted in severe frost. Activation of buds by placing cut setts for 24 h in fresh cow dung improved germination [1].
- **Altering planting time**: In Hualien area (Taiwan) for nearly 5 months (November to March), the mean temperature remained $<20\,^{\circ}C$, which affected growth and yield if cane was planted in August. Preponing planting (to June or July) ensured abundant sunshine and higher temperature that improved cane and sugar yield [102].
- **Frequent post-monsoon irrigation**: Frequent post-monsoon irrigation reduced the damage due to frost [11]. This is, however, helpful in light to moderate frost.
- **Reducing post-frost losses**: All the heavily frosted canes, as they do not recover, should be milled as soon as possible. In a field study conducted at Jalandhar, India, frost-susceptible sugarcane variety CoJ 70 was sprayed with 50 or 100 ppm of gibberellic acid before the onset of low-temperature stress. The results showed a significant increase in total chlorophyll, soluble sugars, proteins, and nitrate reductase activity in the leaf tissues and sucrose, purity, and commercial cane sugar percentage in the cane juice in the post-frost period [103]. In Florida, Ulloa and Anderson [104] have observed that application of calcium silicate improved tolerance of sugarcane to freeze damage.

35.2.2.4.2 Overcoming Poor Sprouting in Winter-Initiated Ratoons

- **Avoidance of low temperature for the exposed stubble**: In winter-initiated ratoons, poor sprouting could be overcome by spreading a trash cover [105–107], or by spreading a polyethylene cover [108] over the soil before the advent of low temperature phase. These may be removed after the low temperature phase is over [107, 108].
- **Biochemical manipulations using growth regulators/nutrients**: Plant growth regulators and certain nutrients have been used for improving stubble sprouting and productivity of winter-initiated ratoon crops of sugarcane (Table 35.1).
- **Maintaining optimal clump population**: Attempts should be made to maintain an optimal clump population (29 000 clumps ha^{-1}) in subtropical India [119]. Gaps should be filled by using stubble dugout from other fields, pre-germinated settlings, or the sprouts from the clumps of the same variety in the same field. Gaps should be filled in the early tillering phase as late transplants do not keep pace with the development of normal tillers [120].

Table 35.1 Use of plant growth regulating substances and nutrients for improving stubble sprouting and yield of winter-initiated ratoon crop.

S. No.	Country	PGR/nutrient	Reference
A. Application to the freshly harvested stubble (after harvest of plant crop)			
1.	Taiwan	Cycocel (10 kg a.i. ha^{-1})	[109]
		IBA (200 ppm) and Ethrel (1000 ppm)	[110]
2.	India (subtropical)	Cycocel (8 kg a.i. ha^{-1}); IBA (10 ppm)	[111]
		Cycocel (5 kg a.i. ha^{-1})	[112]
		Ethrel (2500 ppm) + gamma HCH (1 l ha^{-1})	[113]
		Ethrel (500 ppm); IBA (100 ppm) and TIBA (50 ppm)	[108]
		Formulation containing mixture of vitamins	[114]
B. Application to the preceding plant crop (15 days before harvest of plant crop)			
1.	Taiwan	Ethrel (1.44 kg a.i. ha^{-1})	[115]
2.	India (subtropical)	Ethrel (200 ppm) + Urea (4%)	[116]
		Glyphosate (0.16 kg a.i. ha^{-1})	[117]
		Application of 66 kg K ha^{-1} with last irrigation a month before harvest	[118]

35.2.2.5 High Temperatures

High temperature as a stress affecting sugarcane productivity could be visualized as (a) cultivation of sugarcane in hot and dry areas (as in Sudan and Malawi), (b) experiencing relatively higher temperatures at certain growth stages (early growth stage in a late summer planted crop, overstands), (c) pre-harvest burning of sugarcane, dumping bagasse, trash, and so on, and (d) gradually increasing temperatures due to global climatic change. In hot and dry areas, cultivation of tolerant varieties will be desirable [1]. Experience at the Indian Institute of Sugarcane Research, Lucknow, India, has indicated that the use of trash mulch in hot summer months reduced the soil temperature by 2–3 °C and thus may sustain the functioning of roots. As a result of late planting of sugarcane in summer months (April and May), owing to high temperatures, low RH, and scanty rainfall, the crop experiences heat stress and germination and early growth of the crop are affected. Irrigation immediately after planting improves germination and cane yield [121].

35.2.2.6 Nutrient Stress

The lopsided use of fertilizer NPK has led to both an imbalance in nutrient use and a decreased availability of secondary and micronutrients. Deficiency of S and some of the micronutrients has been well recognized and deficiency of K is increasing in Indian soils. Problems related to nutrients in sugarcane are chlorosis (a widespread nutritional malady), imbalance of nutrients, high sheath moisture (in peninsular India), and certain nutrient interactions (Zn deficiency is commonly encountered following P addition when cane is grown after paddy; excessive P fertilizer induced Zn deficiency in ripe canes; Mn deficiency is associated with soils high in Mg, Ca, and N; nutrient deficiencies, especially of K, may cause more uptake of Ca and lead to processing problems; cane grown in laterite soils of Orissa have higher amount of colloidal silica in juice [1]. Besides, certain abiotic stresses also induce nutrient deficiencies. In rain-fed areas, which experience frequent drought spells, multi-nutrient deficiency (Zn and K) is common. An optimal nutrient supply (N, P, K, and Zn) ensures deeper root system, helps in better adaptation of crop to sustain productivity under such conditions [50]. In saline–alkaline soils, deficiency of P, Fe, and Zn is of common occurrence, and remedial measures for this have been suggested by Zende and Hapase [89] as mentioned above.

Application of soluble silicates is beneficial in areas where sugarcane growth is affected by absorption of excess amounts of heavy metals like Mn [122].

35.3 Breeding Varieties Tolerant to Abiotic Stresses

35.3.1 Tolerance/Resistance to Abiotic Stresses in *Saccharum* Spp. and Related Genera

Earle [123] has mentioned that *Saccharum sinense* clones have strong root system and great power and ability to thrive on salt-affected soils. *S. barberi* clones exhibited

Table 35.2 Sugarcane varieties tolerant to various abiotic stresses.

Variety	Characteristics
Batjan	Vigorous growing, high tonnage, adapted to medium and poor soils
Cavengirie	Good yield on poor, dry lands
Co210	Adapted to hard, dry land, and waterlogging
Co 281	Cold resistant
C 46	Grew well in sandy *sabana* lands and adapted to shallow lime soils
Daniel Dupont	Early ripening and adapted to high altitudes
D 109	Withstood unfavorable conditions
D 117	Withstood salt-affected soils
EK 28	Thrived fairly well on a variety of soils
POJ 36; POJ 2725	Well adapted to poor and exhausted lands
POJ 213	Adapted to wide range of soils and withstood well wetlands and lack of drainage
POJ 2727	Adapted to dry, rocky lands
Uba	Maintained sucrose content and purity on alkaline soil than on acid soils

Source: Ref. [123].

considerable resistance to cold; thus, they are adapted to subtropical climates. He has also mentioned some of the then prevailing varieties that were tolerant/resistant to some of the abiotic stresses, poor soils, high altitudes, and so on (Table 35.2). These could be utilized as parents for breeding varieties tolerant to abiotic stresses.

In an evaluation of 28 clones of *S. spontaneum* for their tolerance to artificially created waterlogging, two clones, namely, SES 334 (Assam) and SES 340 (Manipur), had the best growth, tillering, and lush green foliage. There was a characteristic development of a large matrix of fibrous, negatively geotropic roots. On the basis of these observations, Srinivasan and Rao [15] suggested that these clones could be utilized as genetic donor to evolve waterlogging-tolerant sugarcane varieties. During waterlogged conditions (6 months of flooding), all the clones of *S. officinarum* quickly died. *S. barberi*, *S. sinense*, *Sclerostachya*, and *Erianthus* spp. were all susceptible but survived flooding. Several clones of *S. spontaneum*, *S. robustum*, and *Narenga* spp. were tolerant to waterlogging. It was generalized that clones with profuse development of fibrous, floating, negatively geotropic roots with aerenchyma were tolerant to waterlogged conditions. Such a trait, present in *S. spontaneum*, could be transmitted through crossing [16].

In the *World Germplasm Collection* maintained at SBI research station, Cannanore, India, following clones/varieties of various *Saccharum* spp. have been reported to be resistant to salinity [124]:

S. barberi: Katha, Coimbatore, Kewali-14-G, Khatuia-124, Kuswar, Ottur; Lalri, Nargori, and Pathri.
S. sinense: Khakai, Panshahi, Reha, and Uba-Seedling.
S. robustum: IJ-76-422, IJ-76-470, 28-NG 251, 57-NG-201, 57 NG -231, NG-77-34, NG-77-55, NG-77-136, NG-77-160, Ng-77-167, NG-77-170, NG-77-221, and NG-77-237.

Studies conducted at Karnal indicated that sugarcane variety Co 453 could be utilized as a best donor for imparting tolerance to salinity–alkalinity. Sugarcane variety Co 312 has also been used as a parent for imparting drought tolerance in sugarcane variety Co 87263 [125].

Generally, greater low-temperature tolerance was observed in high-fiber commercial clones [126] and regional selections of *S. spontaneum*, *S. sinense*, and allied genera, for example, *Miscanthus* [127]. Brandes [128] identified highly cold-tolerant clones of *S. spontaneum*; this trait was, however, lost through hybridization and backcrossing with *S. officinarum*. Roach and Maynard [129] found 3.9% of the progeny of 28 crosses between *S. spontaneum* and *S. officinarum* were freeze tolerant, but after backcrossing the frequency of tolerance in the progeny dropped to 0.5%. Dunkelman and Breaux [130] were, however, able to maintain an acceptable level of tolerance through two backcrossings. On the basis of this observation, Moore [131] concluded that if tolerant clones were used as recurrent parent, greater cold tolerance could be obtained in commercial varieties. Certain varieties of *S. spontaneum* were resistant to damage by prolonged low temperatures. One strain *Arbington Va Winter* remained green throughout [128]. At Houma in Louisiana (USA), sugarcane breeding strategies include (i) evaluation of parents and progeny for resistance traits such as cold tolerance and (ii) backcrossing and recombining progeny in a systematic manner that will concentrate genes associated with this trait. In 2002, 2169 progeny from 11 interspecific crosses between 10 *S. spontaneum* clones and 6 commercial varieties were evaluated. In February 2003, 10 crosses from SES 234 × LCP 85–384 were identified as expressing cold tolerance after a 3 h −5 °C freeze on January 2003. These selections expressed minimum levels of dead leaf tissue, few dead terminal buds, and no stalk damage. These were verified as interspecific hybrids using microsatellite DNA marker [132].

In 1982, Sikkim Himalayas were surveyed and clones belonging to *Miscanthus nepalensis*, *Erianthus fulvus*, *E. arundinaceus*, *E. procerum*, and so on were collected. The high-altitude forms of *M. nepalensis* and *E. fulvus* did not survive at Coimbatore. However, these were successfully established at the IARI Regional Research Station, Wellington [46]. Few clones of Co 7201 × *Erianthus* spp. continued to grow in winter at Karnal (in subtropical India) and some of these were both cold tolerant and resistant to red rot [133]. These could be utilized to impart low-temperature/cold tolerance.

Among other varieties developed from the cross of Co 281 (as a pistil parent) to POJ 2878 (as pollen parent) were PR 980 (widely used as a parent to impart drought and frost resistance), PR 1000 (used as a parent for imparting sucrose to the progeny), PR 1013, PR 1016, and PR 1028 (adapted to poorly drained and waterlogged conditions). In Reunion, it was used as a pistil parent to evolve variety R 397 [134]. At Coimbatore, Co 312 has been used as a parent to impart drought tolerance in variety Co 87263 [125]. In Mexico, in sugarcane breeding programs, emphasis is given on selection of varieties with deep root system and drought resistance, good proline under nonirrigated conditions, and resistance or tolerance to mosaic, rust, and smut.

They also look for varieties adapted to as high as 1300 amsl and annual rainfall from 300 to 2500 mm [135].

Saccharum species and related genera that may impart tolerance/resistance to abiotic stresses and nutrient use efficiency in sugarcane are given in Table 35.3.

Table 35.3 *Saccharum* species and related genera that may impart tolerance/resistance to abiotic stresses and nutrient use efficiency in sugarcane.

Characteristic	Genera/species	References
Tolerance/resistance to drought	*S. spontaneum*, *Narenga* spp. [a] *Erianthus* spp.	[136]
Tolerance/resistance to waterlogging	*S. robustum* *S. spontaneum*	[136]
Tolerance/resistance to cold (performance at high altitudes)	*Miscanthus* spp. *Miscanthus nepalensis*, *E. fulvus* *S. spontaneum* *S. barberi*	[46, 123, 128, 136, 137]
Tolerance/resistance to salinity	*Erianthus* spp. *S. barberi*, *S. sinense* *S. robustum*	[124, 137]
High nutrient use efficiency	*S. spontaneum* (IK 76-20, SES 24, IS 760164) *S. robustum* (51 NG 27) *S. sinense* (Khadaya) *S. officinarum* (UB-16)	[138]
Low nutrient requirement	*S. spontaneum*, *Erianthus* spp.	[136]
Robust growth under low input conditions	*Erianthus* spp.	[137]

a) When *E. arundinaceus* was used as a pollen parent, there was no significant reduction in sucrose content [134].

Some of the desirable features of indigenous canes growing in India, possessing tolerance to some abiotic stresses, which could be utilized in directed breeding are mentioned in Table 35.4 [139]. Of these only four (Chunnee, Katha, Saretha, and Kansar) have been utilized in breeding. Other indigenous varieties need to be utilized for developing sugarcane varieties tolerant to abiotic stresses.

This approach seems to be promising as the use of *Mandalaya*, a *S. spontaneum* clone from Burma, led to the success of Australian "Early CCS Canes Programme" and the use of another *S. spontaneum*, US56-15-8, led to development of LCP 85–384, a high yielding, high sugar, early ripening, less N requiring, and cold-tolerant variety of Louisiana [140].

Table 35.4 Desirable features of indigenous canes growing in India that could be utilized in directed breeding.

Variety	Tolerance to abiotic stress	Other associated desirable features
In subtropical India		
Chin, Chunnee, Raksi, Burra Chunnee, Baraukha	Flooding	Early ripening (harvested in December/January), high fiber, high sucrose
Agoul	Grew with less water (and manure)	
Hemja	Well adapted to early drought and late waterlogging	Heavy tillering, heavy yielder, high sucrose and purity, resistant to red rot and borers
Maneria, Chinia	Withstood waterlogging	Grew in irrigated areas, erect, high tillering, good sucrose content. Maneria was also tolerant to borers
Khari	Drought and waterlogging	Good germinator, heavy yielder, good ratooner
Sewari	Flooding	Early ripening
Katha	Wide adaptability to drought, rainfed, flooding, hot and dry climate, and to a lesser extent to frost	Early ripening, thin excellent tillering
Lalri	Frost	Hardy, good tillering, resistant to red rot
Khari, Ikhri, Khagri[a)]	Drought and waterlogging	
In tropical India		
Kalkya, Khadiya, Bansi, Sunnabile	Drought	Heavy tillering and ratooning ability
Nannal	Drought	

Source: Abridged from Ref. [139].
a) *Khagri* was able to grow 6 feet under water for over 3 months.

35.3.2
Varieties Developed/Identified Tolerant to Abiotic Stresses in India

In India, the All India Coordinated Research Project on Sugarcane has released some sugarcane varieties tolerant to abiotic stresses for commercial cultivation in various zones in India (Table 35.5).

Tew [142] and Shrivastava and Srivastava [1] have mentioned varieties tolerant to abiotic stresses used in various parts of sugarcane growing areas in the world.

Some of the sugarcane varieties possess *multiple abiotic stress tolerance* (Figure 35.2). There is need to elucidate physiological and biochemical characteristics associated with these varieties. Breeding programs must be tailored to breed such varieties, as in

Table 35.5 Sugarcane varieties (tolerant to abiotic stresses) released by the AICRP (Sugarcane) in India.

S. No.	Variety	Year of release	Cane yield (ton ha^{-1})	Sucrose (%)	Maturity group	Reaction to disease	Tolerance to abiotic stress
Peninsular zone (Madhya Pradesh, Gujarat, Karnataka, Kerala, interior Andhra Pradesh, and plateau region of Tamil Nadu)							
1.	Co 94008 (Shyama)	2002	119.8	18.3	Early	MR-red rot	DR, salinity, WA
2.	Co 8371 (Bhima)	2000	117.7	18.6	Mid-late	R-smut	DR, WL
3.	Co 87025 (Kalyani)	2000	98.2	18.3	Mid-late	R-smut	DR, WL
4.	Co 87044 (Uttara)	2000	101.0	18.3	Mid-late	R-smut	DR
5.	CoM 88121 (Krishna)	2000	88.7	18.6	Mid-late	R-smut	DR, MQL
6.	Co 91010 (Dhanush)	2000	116.0	19.1	Mid-late	R-smut	DR,RF
7.	Co 99004 (Damodar)	2007	116.7	18.8	Mid-late	MR-red rot	DR, salinity
8.	Co 2001-13 (Sulabh)	2009	108.59	19.03	Mid-late	MR to red rot, smut and wilt	DR, salinity stress
9.	Co 2001-15 (Mangal)	2009	112.99	19.37	Mid-late	MR to red rot and smut	DR, salinity and lodging
East-coast zone							
10.	CoC 01061	2006	110.8	17.4	Early	MR to red rot	DR
North-central zone (Eastern Uttar Pradesh, Bihar, and West Bengal)							
11.	Co 87263 (Sarayu)	2000	66.3	17.4	Early	R-smut, red rot	WL, RF, LIC
12.	Co 87268 (Moti)	2000	78.9	17.5	Early	R-smut, red rot	DR,WL, high soil pH
13.	CoSe 96234 (Rashmi)	2002	64.1	17.9	Early	MR-red rot	Stress conditions, in general
14.	CoSe 96436 (Jalpari)	2002	67.1	17.7	Mid-late	MR-red rot	WL
15.	CoLk 94184 (Birendra)	2008	76.0	18.0	Early	MR to red rot	DR, waterlogging
North-west zone (Punjab, Haryana, Rajasthan, western, and central Uttar Pradesh)							
16.	CoH 92201 (Haryana-92)	2000	70.0	18.2	Early	R-red rot	LPC
17.	CoS 95255 (Rachna)	2002	70.5	17.5	Early	MR-red rot	LPC
18.	CoPant 90223 (Pant 90223)	2000	73.3	18.5	Mid-late	MR-red rot	DR, WL, LT
19.	CoPant 93227 (Pant 93227)	2002	75.4	17.3	Mid-late	R-red rot	LIC, suboptimal environments

(*Continued*)

Table 35.5 (*Continued*)

S. No.	Variety	Year of release	Cane yield (ton ha^{-1})	Sucrose (%)	Maturity group	Reaction to disease	Tolerance to abiotic stress
20.	Co 86249 (Bhavani)	2000	104.2	18.7	Mid-late	R-smut, red rot	WA
21	Co 98014 (Karan-1)	2007	76.3	17.6	Early	MR to red rot	DR, waterlogging
22.	CoPant 97222	2007	88.2	18.2	Mid-late	MR to red rot	DR, waterlogging, salinity
23.	CoH 119	2005	82.8	17.5	Mid-late	—	DR

Source: Up to 2002 from Ref. [141]; others by courtesy of Dr. O.K. Sinha, Project Coordinator, AICRP (Sugarcane), Lucknow.
DR: drought tolerant; WL: waterlogging tolerant, RF: suitable for *rain-fed* conditions; WA: suitable for wider adaptability; LIC: suitable for low-input conditions; MQL: maintains juice quality longer; LPC: suitable for late planted conditions.

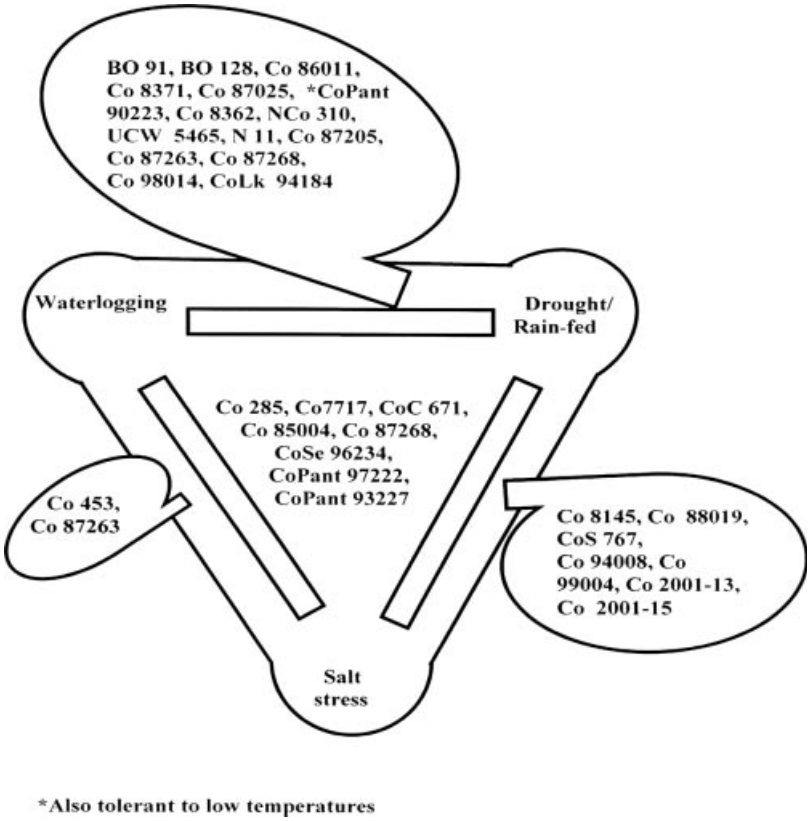

Figure 35.2 Sugarcane varieties exhibiting multiple –abiotic stress tolerance.

nature, more than one stress often occur together or the prevalence of one stress may accentuate or aggravate the other to further augment the losses.

35.4 Molecular Approaches

Selection and breeding using wide crosses are the best short-term approach for developing a stress-tolerant sugarcane variety. However, new molecular and biotechnological approaches may go a long way in developing transgenic plant for tolerance to a particular stress or multiple stresses. Epstein and Rains [143] have outlined the steps for a general program for developing a stress-tolerant plant using biotechnological/molecular approaches. It includes cloning of stress tolerance genes from microorganisms, recombinant DNA analysis of the structure of genes required for stress tolerance, development of a plant gene vector for stress tolerance gene transfer, cloning of stress tolerance genes using recombinant DNA technique, transferring these genes (segment) into a prevailing good commercial variety,

selection of stress-tolerant mutant plants using tissue culture and hardening of these plants, and evaluating their field performance.

There are many possibilities for the manipulation of genes to impart/transfer the stress resistance trait in high-yield varieties. Structural and functional characterization of environment stress-induced genes has contributed to a better understanding of how the plants respond and adapt to different abiotic stresses. Studies have shown that specific proteins, "stress proteins," accumulate in response to imposition of stress conditions, namely, drought, low temperature, high temperature, oxidative stresses, and so on.

Grover *et al.* [144] have reviewed various experiments conducted in the molecular biology of abiotic stress responses, which are triggered at different levels of hierarchy of the cellular organization, in different systems, namely, lower and higher plants and microbes. The molecular analysis of the stress responses has been carried out at the level of stress proteins, stress genes, stress promoters, transacting factors that bind to stress promoters, and signal transduction components involved in bringing about the stress responses. They have listed a large number of genes/proteins associated with tolerance to water stress, salt stress, anaerobic stress, high- and low-temperature stress, heat shock, and so on. The functional relevance of the stress-associated genes is being tested in different trans-systems in plant species. Some of these genes might be utilized in times to come to develop transgenic plants tolerant/resistant to various abiotic stresses. Traditional plant breeding techniques will be suitable for transfer of traits of stress tolerance. Such studies are also in vogue in sugarcane. These may help in sustaining sugarcane and sugar productivity under abiotic stress conditions in times to come.

35.4.1 Drought

Development of drought-tolerant sugarcane varieties through genetic engineering is a serious option for managing drought. Some candidate genes for drought tolerance have been identified, for example, dehydration-responsive transcription factor (DREB) genes that mediate transcription of several genes in response to water stress, genes encoding enzymes of the biosynthetic pathways of different osmolytes such as proline, glycine betaine, sorbitol, heat shock proteins (HSPs), late-embryogenesis proteins, and RAB (responsive to abscisic acid) proteins. Besides, osmotin, choline oxidase, annexin, and genes encoding superoxide radical (O^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) also have potential in drought tolerance [4].

Trehalose synthase (*Tsase*) gene (from *Grifola frondosa*) transferred into a sugarcane hybrid by *Agrobacterium tumefaciens* (strain EHA 105) imparted resistance to osmotic stress. Some of the transgenic plants showed improved tolerance to osmotic stress. After transplanting in MS medium containing 17.4% PEG 8000, the transgenic plants demonstrated improvement in tolerance to osmotic stress [145]. The use of a novel sugarcane ethylene-responsive factor, SodERF3, could be a valuable tool to assist the manipulation of sugarcane plants to improve their stress tolerance [146]. Increased expression of a gene encoding a peroxidase has been reported by Rodrigues *et al.* [147] in a drought-tolerant sugarcane cultivar. Catalase (CAT) enzyme is responsible for the reduction of H_2O_2 to H_2O and O_2, and a decline in peroxidase

activity is considered a limiting step to neutralization of reactive oxygen species (ROS) in sugarcane [148].

The cDNA-AFLP technique was used to identify differentially expressed genes in two sugarcane cultivars that had previously been classified as tolerant (TSP-054) or susceptible (TCP02-4589) to drought [149]. A total of 23 transcript-derived differentially expressed fragments (TDFs) were observed and 5 of these revealed high identity with stress-related genes previously reported in sugarcane, sorghum, rice, and corn. One TDF that was upregulated in the tolerant cultivar after 12 days of stress exposure showed complete identity with a drought-inducible gene (SoDip22) previously identified in sugarcane. A TDF that was downregulated in the susceptible cultivar also had high identity (87%) with a putative pentatricopeptide expressed in rice. After rehydration, the normal gene expression pattern was reestablished, thus demonstrating the ability of sugarcane to respond rapidly to changing environmental conditions.

Real-time reverse transcription–PCR profiling of selected expressed sequence tag (EST) clusters identified several sugarcane clusters that showed differential expression in response to biotic and abiotic stress conditions [150]. Twenty-five stress-related clusters have shown more than twofold relative expression during water deficit stress. All clusters that showed homology to osmotic stress or dehydration stress-associated proteins were significantly upregulated. A very high (~13-fold) induction during 9 h of dehydration stress was noted in the case of a salt stress-inducible Bzip protein. These might be delayed action genes working in the related pathways. Some clusters showed higher expression during 3 h and then a decline during 6 h and again expressed to significantly higher levels at later stages (9 h). This pattern of expression and delayed response of certain genes could be attributed to their tight regulation via other candidates involved in the pathway.

Iskandar *et al.* [151] analyzed expression of 51 genes implicated in abiotic stress in relation to sucrose accumulation by studying tissues of internodes of mature and immature stalks in a high sucrose accumulating sugarcane cultivar in normal and water stress conditions to compare effects of sucrose accumulation and water deficit. They identified a subset of stress-related genes including genes encoding enzymes involved in amino acid metabolism, a sugar transporter, and a transcription factor that are potentially associated with sucrose accumulation in stalk. Expression of these stress response genes in plants that were under water deficit stress revealed a different transcriptional profile that was correlated with sucrose accumulation. Besides, genes with homology to late-embryogenesis abundant (LEA)-related proteins and dehydrin were strongly induced under water deficit, but this did not correlate with sucrose content. The study indicated that while there was a change in stress-related gene expression associated with sucrose accumulation, different mechanisms are probably responding to the water-deficit-induced stress.

Upregulation of genes encoding for polyamine oxidase, cytochrome-*c*-oxidase, *S*-adenosylmethionine (SAM) decarboxylase, and thioredoxins, which directly or indirectly participate in the regulation of the intracellular redox status, have been demonstrated in sugarcane under drought stress [152], and may probably contribute to water-deficit-induced stress tolerance. Stress cluster 1 (putative oxidative stress

responsive) and stress cluster 15 (70 kDa heat shock protein) showed upregulation during 3–6 h of dehydration stress, but later on their expression decreased [150]. These genes might provide tolerance to stress in its initial stages. The accumulation of the osmolytes trehalose and proline also contribute to reduction in damage caused by accumulation of ROS and provide enhanced tolerance to drought [13, 153, 154]. Some of the sugarcane genes expressed under water deficit stress might be involved in the pathways that lead to the production of such osmoprotectants.

Jangpromma *et al.* [155] reported an unknown 18 kDa protein (p18) along with other stress-inducible proteins to be highly expressed in sugarcane leaves under drought conditions. The 2D-PAGE patterns of proteins were compared with those expressed in drought tolerant K86–161 and drought-susceptible Khon Kaen 1 cultivars. Mass spectrometry and comparison with known sequences, in the database, revealed that the proteins expressed only in stressed K86–161 were serine protease inhibitor and the one similar to replication protein A1. A group of proteins upregulated in K86–161 includes *S*-adenosylmethionine decarboxylase proenzyme (SAM), ubiquitin, and p18. Higher levels of chlorophyll and SOD in drought-tolerant than those in drought-susceptible sugarcanes are in agreement with a high level of p18 expression in drought-tolerant sugarcane. An accumulation of p18 appears to be in response to water deficit.

In plants, micro-RNAs are involved in the regulation of plant development and nutrition, responses to biotic and abiotic stresses, signal transduction, and protein degradation. The possible role of sugarcane microRNAs in the regulation of drought stress is being studied by Rojas *et al.* [156].

35.4.2 Waterlogging

Although physiological adaptations of crop plants to waterlogging/flooding (hypoxia or a anoxia) have been studied [157, 158], the molecular basis of such adaptations has not been completely understood [159]. However, some progress has been made in identifying genes and gene products induced during low oxygen conditions [159]. Low O_2-induced genes are characterized by the presence of an anaerobic response element (ARE) in the promoter [160]. It has been observed that about 20 anaerobic polypeptides are synthesized under hypoxia [161]. These may confer tolerance to hypoxia. A transcription factor, AtMYB2, which is induced by dehydration, salt stress, and exogenously applied ABA [162], is also induced by hypoxia. It is induced in root tissues and its induction coincides with the induction of ADH1Mrna. Attempt has also been made to use this transcription factor to affect the expression of anaerobic polypeptides (ANPs) produced during hypoxia. Dennis *et al.* [159] are of the opinion that AtMYB2 gene may enable the transgenic plants developed to recognize anaerobic conditions earlier, produce ANPs, and impart tolerance to hypoxia.

In an effort to elucidate the genes underlying plant responses to inundation, subtractive cDNA libraries were made from a highly adapted waterlogging-tolerant sugarcane variety, BO 91, grown under stress and normal conditions to identify the genes differentially expressed in waterlogging stress [163]. Major groups of ESTs were

related to stress (15%), catalytic activity (13%), cell growth (10%), and transport-related proteins (6%). A few stress-related genes identified were senescence-associated proteins, dehydration-responsive family proteins, heat shock cognate 70 kDa protein, and so on.

Plants with increased tolerance to waterlogging were obtained from callus lines that had been selected for resistance to ethionine, the toxic analogue of methionine, and to anaerobic conditions. Such identified plants may be used as donors to impart resistance to waterlogged conditions [164].

35.4.3 Salinity

Response of genotypes to callogenesis from leaf explants varied considerably under salt stress. A sugarcane genotype, BF-162, exhibited better regeneration of plantlets at all the salt levels than other varieties. The genotype CP 43/33 and *Triton* were relatively more sensitive to salt stress and regenerated only up to 0.9 g l^{-1} NaCl [165]. RAPD analysis of six salt-tolerant clones, regenerated from calli adapted to high salt medium, showed a DNA profile distinct from that of the original genotype. A unique DNA fragment of 995 bp, found only in salt-tolerant clones, showed sequence similarity to the genes involved in stress tolerance [166]. Embryogenic sugarcane (*S. officinarum* L.; cv. CoC 671) calli treated with different levels of NaCl accumulated higher amounts of free proline and glycine betaine [167]. Although the leached and retained Na^+ contents increased, the retained K^+ content decreased with increasing levels of NaCl. The growth retardation and reduced cell viability were associated with a conspicuous increase in Na^+ and a corresponding decline in K^+ concentrations. Such a mechanism implied that sugarcane could be considered as a Na^+ excluder. The accumulation of salt ions and osmolytes could play an important role in osmotic adjustment in sugarcane cells under salt stress. Moreover, NaCl priming treatments improved several aspects of plant growth and exhibited better tolerance of sugarcane seedlings to salt stress [168].

Accumulation of toxic ions in plant tissues modulates the levels of primary and secondary metabolites, which may be related to salinity tolerance [169]. Sugarcane clones exposed to salinity levels at the formative stage displayed a general tendency to accumulate Na^+ and Cl^- and little K^+, though the salt-tolerant clone CP 4333 accumulated less Na^+ and more K^+ compared to the salt-sensitive clone HSF 240, and thus showed a higher K^+: Na^+ ratio. Soluble phenolic, anthocyanin, and flavone levels were 2.5, 2.8, and 3.0 times greater in CP 4333 in comparison to HSF 240. The decrease in net rate of photosynthesis and most secondary metabolites in salt-sensitive clone indicated their relation with increased salt tolerance of sugarcane.

35.4.4 High-Temperature Stress

In recent times, higher temperatures are becoming more important as an abiotic stress. Studies have shown that enhanced synthesis of an oxidant by plant tissues

might increase tolerance to heat stress in soybean [170], and increase in saturated fatty acids of membrane increased their melting temperatures and imparted heat tolerance in *Arabidopsis* spp. [171, 172]. Most common molecular response of plants exposed to heat stress is the expression of heat shock proteins, which have a fairly wide range of molecular masses (10–250 kDa). They associate with various cellular structures or organelles to provide protection and act as molecular chaperones [173]. The dehydrin proteins (DHNs) are among other proteins that are classified as a group of late-embryogenesis abundant proteins, referred to as LEA group II, and typically accumulate late in embryogenesis or in vegetative tissues in response to environmentally imposed dehydrative forces, such as drought, salinity, and freezing [174]. Like most mesophytic species, sugarcane also shows sensitivity to supraoptimal growth temperatures despite the fact that it has higher temperature optimum compared to C3 species [175]. Expression of HSPs in the cultured sugarcane cells was noticed as a heat tolerance response [176]. In sugarcane seedlings subjected to heat stress, Wahid and Close [177] observed expression of three heat stress-induced DHNs with an apparent molecular mass of 21, 23, and 27 kDa under heat stress (48–72 h). The independent expression of these DHNs with reference to the changes in water relations of leaves suggested that they have a definitive protective role like other heat stress proteins.

There is need to characterize the genetic variability of specific HSPs across wide range of germplasm of *Saccharum* and related genera. Biochemical studies are needed to elucidate as to how these HSPs protect or allow restitution from high-temperature stress. HSPs appear to maintain the conformation of other protein structures [178]. The temperature range over which the apparent Michaelis–Menten constant for CO_2 (K_m) is minimal and stable is termed the thermal kinetic window [179]. There is a need to establish *thermal kinetic window* for sugarcane.

35.4.5 Low-Temperature Stress

For increasing productivity by enhancing stress tolerance, identifying relevant genes and characterizing their functions and regulation in response to low-temperature stress is important. Nogueira *et al.* [180] have identified 34 cold-inducible expressed sequence tags (ESTs) of which 20 were novel cold-responsive genes including cellulose synthase, ABI 3-interacting protein 2, a negative transcription regulator, and a phosphate transporter. Besides, 25 ESTs have also been identified that were downregulated during exposure to low temperatures. On the basis of the expression profiles of the cold-inducible genes and the data-mining results, sugarcane has two putative dehydrin-like proteins (WCOR410b and DHN2). These proteins could stabilize macromolecules and/or protect membranes against chilling damage [181]. Sugarcane ESTs encoding PPDK and NADP-ME proteins were induced by cold exposition, suggesting a possible maintenance of photosynthesis, even at low temperatures. It is possible that sugarcane putative antifreeze proteins can confer cellular membrane protection, reducing chilling injury.

In response to prolonged winter chill (PWC)-induced low-temperature stress, the tolerant sugarcane cultivars maintained relatively higher chlorophyll, carotenoid, N, and micronutrient and proline contents than the susceptible ones. Increased peroxidase activity was observed in the leaf and apical meristem in response to PWC-induced low-temperature stress along with the expression of some new isozyme bands of low molecular weight [182]. The photosynthetic rate is severely reduced under chilling temperature conditions [183]. The activity of photosynthetic enzymes such as sucrose phosphate synthase [184], NADP-malate dehydrogenase, and pyruvate orthophosphate dikinase activities [185] decreased when a cold-sensitive cultivar of sugarcane (*Badila*) was subjected to chilling temperatures (10 °C). The biochemical changes in response to cold treatment led to increased aspartate and alanine levels in the leaves of the cv. *Badila* plants. A complex antioxidant system has been suggested to act as a protective mechanism against chilling injury in sugarcane [186].

35.4.6
Nutrient Stress

The importance of K and P as essential elements for plant is undisputable; however, excess K [187, 188] and excess P [189] can result in a significant depression in sucrose concentration in sugarcane.

Sequence clusters homologous to plant high-affinity phosphate transporter genes have been identified from SUCEST data base. Identification and expression of genes associated with nutrient uptake and distribution may lead to the development of enhanced nutrient use efficiency of sugarcane, and economize the application of fertilizers to sugarcane crops [190].

35.4.7
Heavy Metal Stress

The use of tannery and industrial effluents, municipal waste-based composts, and biosolids' application in sugarcane fields and production of sugarcane on metal-polluted fills might create the problem of heavy metal stress for sugarcane cultivation [191, 192]. Pollution caused by pesticides, fertilizers, sewage sludge, industrial residues, and herbicides, which contain different concentrations of toxic metals, may severely affect sugarcane growth and metabolism [193]. Jain *et al.* [194] observed inhibitory effects of nickel at high levels (50 and 100 ppm Ni) on shoot and root growth, mitotic efficiency, metabolic attributes, and nutrient uptake of sugar cane (*Saccharum* spp. hybrid CoLk 8102). High concentrations of zinc (65 and 130 mg l^{-1}) were shown to increase lipid peroxidation in sugarcane, thereby affecting membrane integrity of leaves, root growth, and mitotic efficiency. The interference of Zn in normal mitosis could be related to an inhibition of DNA synthesis [195]. Higher chromium concentrations inhibited bud germination of sugarcane and induced chlorosis of young emerging settlings that turned necrotic at later stages. Biochemical studies revealed a decline in specific activity of catalase and an increase in

reducing sugar content in settlings supplied with chromium [196]. Mitotic efficiency of root tip cells of chromium treated setts declined and the frequency of aberrant mitotic phases increased *pari passu* to the increasing chromium concentration [197]. Root meristem assay of sugarcane settlings grown on crude and digested spent wash showed a detrimental effect on mitotic efficiency, induction of *de novo* chromosomal aberrations, namely, clump formation, chromosome stickiness, laggards, and micronuclei formation, and higher number of chromosomal abnormalities compared to those of control conditions [198].

35.5 Abiotic Stresses could also be Beneficial

Abiotic stresses are not always harmful; sometimes, these could be beneficial also. For example, in sugarcane the advent of low temperatures (which otherwise are unfavorable for growth) hastens ripening. When relatively higher temperatures prevail during ripening, cane does not ripen properly. Under these conditions, giving moisture stress by withholding water promotes ripening. Inducing drought hardiness by treating seed cane with salt solutions provides tolerance to drought to some extent. Flooding also controlled certain insect pests such as giant moth borer, *Castina licoides* [199], *Ligarus* grubs, and *Melanotus* [200]. In the scenario of global climate change, the increasing temperatures may not adversely affect the sprouting of the winter-initiated ratoon crop and its productivity.

35.6 Concluding Remarks

On the basis of the above discussions, one may conclude that the physiological, breeding, and molecular approaches will go a long way both in improving abiotic stress tolerance of sugarcane and in sustaining its productivity for the benefit of mankind. Compared to certain other less economically important crops, the research on the molecular and biochemical modifications that are involved in adaptation responses to various abiotic stresses in sugarcane are quite limited, and much more needs to be done [201]. Needless to mention that technological gains attained so far have not only to be defended but also to be improved upon to face challenges of climate change, marginalization of arable land, ever-depleting water resources, other abiotic stresses, and above all increasing human population.

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References

1 Shrivastava, A.K. and Srivastava, M.K. (2006) *Abiotic Stresses Affecting Sugarcane: Sustaining Productivity*, International Book Distributing Company, Lucknow, India, pp. 322.
2 Aggarwal, P.K. (2010) *The Hindu Survey of Indian Agriculture 2010*, The Hindu, pp. 64–65.
3 Anonymous (2010) *Coop. Sugar*, **42**, 15–20.
4 Nair, N.V. (2011) *Coop. Sugar*, **42**, 43–52.
5 Shrivastava, A.K., Jain, R., Rai, R.K., and Singh, P. (2009) Compensatory ability in sugarcane, in *Sugarcane: Crop Production and Improvement* (eds S.B. Singh, G.P. Rao, S. Solomon, and P. Gopalasundaram), Studium Press, LLC, USA, pp. 543–556.
6 Humbert, R.P. (1963) *The Growing of Sugarcane*, Elsevier, Amsterdam, p. 710.
7 Webster, P.W.D. and Eavis, B.W. (1971) *Proc. Int. Soc. Sugar Cane Technol.*, **14**, 708–714.
8 Shrivastava, A.K., Singh, K., and Saxena, Y.R. (1985) Production Physiology of Sugarcane: Cultivar Co 1148, Tech. Bull. No. 15, Indian Institute of Sugarcane Research, Lucknow, India, p. 128.
9 Singh, S. and Ramakrishnan, S. (1977) Early growth attributes of thick-stalked versus thin-stalked varieties in relation to drought resistance in sugarcane. *Sugar Cane Breeders' Newslett.*, **40**, 1–4.
10 Evans, H. (1937) *Emp. J. Exp. Agric.*, **5**, 112–124.
11 Gill, H.S. and Singh, S. (1974) *Indian Sugar*, **24**, 261–266.
12 Ho, S.T., Liu, W.F., Chen, Y.H., and Chen, W.S. (1987) *Rep. Taiwan Sugar Res. Inst.*, **116**, 1–6.
13 Guimarães, E.R., Mutton, M.A., Mutton, M.J.R., Ferro, M.I.T., Ravaneli, G.C., and Silva, J.A. (2008) *Sci. Agric.*, **65**, 628–633.
14 Viqueira, L., Costa, I., and Rodriguez, C.R. (1985) *Rev. INICA*, **2** (1), 64–67.
15 Srinivasan, K. and Rao, J.T. (1960) *Curr. Sci.*, **8**, 321–322.
16 Srinivasan, K. and Batcha, M.B.G.R. (1963) *Proc. Int. Soc. Sugar Cane Technol.*, **11**, 571–577.
17 Anonymous (1984) *SBI Newslett.*, **3**, 2.
18 Anonymous (1967) *Salient Research Achievements*, Sugarcane Breeding Institute, Coimbatore, India, p. 13.
19 Rahman, A.B.M.M., Martin, F.A., and Terry, M.E. (1989) *Proc. Int. Soc. Sugarcane Technol.*, **20**, 668–676.
20 Geetha, S. and Balasubramanyam, P. (2000) *Indian Sugar*, **51**, 799–800.
21 Fitch, M.M. and Moore, P.H. (1981) *Plant Physiol.*, **67** (AS.4) 26.
22 Anonymous (1989) *75 Years of Agricultural Research at Anakapalle (1913–1988)*, RARS, Anakapalle, APAU, Andhra Pradesh, India, p. 400.
23 Anonymous (1990) *SBI Newslett.*, **9** (3), 1–3.
24 Nelson, P.N. and Ham, G.J. (2000) *Field Crops Res.*, **66**, 245–255.
25 Torres, A.J., Jiménez, H.R., and Gómez, J.P. (2002) Carta trimestral - CENICAÑA, Centro de Investigación de la Caña de Azúcar de Colombia, 24, 15–16.
26 Shrivastava, A.K., Srivastava, H.M., Darash, R., and Shukla, S.P. (2000) *J. Nucl. Agric. Biol.*, **29**, 42–50.
27 Tai, P.Y.P. and Miller, J.D. (1996) *J. Am. Soc. Sugar Cane Technol.*, **16**, 39–49.
28 Singh, O. and Kaur, S. (1993) *Indian Sugar*, **43**, 491–494.
29 Dutt, N.L. and Vijayasaradhy, M. (1947) *Proc. Indian Sci. Congr.*, **34**.
30 Eggleston, G. and Legendre, B. (2003) *Food Chem.*, **80**, 451–461.
31 Zhang, M.Q. and Chen, R.K. (1993) *J. Fujian Agri. College (China)*, **22**, 23–57.
32 Godshall, M.A., Legendre, B.L., and Buchler, I.P. (1999) *Proc. Int. Soc. Sugar Cane Technol.*, **23**, 335–336.
33 Tai, P.Y.P., Miller, J.D., and Morris, D. (2003) Proc. Annl. Meeting Am. Soc. Sugar Cane Technol. (c.f. *Sugar Cane International*, 2003, Nov./Dec. issue, p. 27).
34 Espinosa, M.A., Chavanne, E.R., Levie, C.A., and Diez, O. (1989) *Revista Industrial Y. Agricola de Tuckman*, **66**, 91–98.
35 Sullivan, C.Y., Norcio, N.V., and Eastin, J.D. (1977) Plant response to high

temperatures, in *Genetic Diversity in Plants* (eds A.R. Muhammed A.K., Sel, and R.C. Von Borstel), Plenum Press, NewYork, pp. 301–317.
36 Anonymous (2008) Annual Report, DARE, Ministry of Agriculture, Government of India and Indian Council of Agricultural Research, Krishi Bhavan New Delhi p. 47.
37 Rajkumar, A.S. and Kambar, N.S. (1999) *Bharatiya Sugar*, **24**, 6–9.
38 Prasada Rao, K.K. (1989) Land marks in sugarcane research at Anakapalle, in *Platinum Jubilee Souvenir (1913–1988)* (ed. K.K. Prasada Rao), Regional Agricultural Research Station, Anakapalle, India, pp. 21–31.
39 Naidu, K.M., Srinivasan, T.R., and Raj, S.M. (1983) Varietal behaviour and management practices for improvement of sugarcane yield and quality under moisture stress. Paper presented at the Workshop of Drought Management of Sugarcane Crop and Drip Irrigation, Trichy, June 18.
40 Perez, Z.F., Romero, E.R., Scandaliaris, J., and Sotillo, S. (1996) *Adv. Agroindustr. (Argent.)*, **16**, 8–11.
41 Stiles, W. and Cocking, E.C. (1969) *An Introduction to the Principles of Plant Physiology*, 3rd edn, Methuen, London, p. 633.
42 Clarkson, D.T. and Hanson, J.B. (1980) *Annu. Rev. Plant Physiol.*, **31**, 239–298.
43 Rutherford, R.S. (1989) *Proc. Annu. Conf. S. African Sugar Technol. Assoc.*, **63**, 136–141.
44 Sato, A.M., Catuchi, T.A., Ribeiro, R.V., and Souza, G.M. (2010) *Acta Physiol. Plant.*, **32**, 1145–1151.
45 Jayabal, V. and Chockalingam, S. (1990) *Coop. Sugar*, **21**, 571–573.
46 SBI (1987) *Research Achievements: 1912–1987*, SBI, Coimbatore, India, p. 121.
47 Annadurai, K., Palaniappan, S.P., Kavimani, R., and Masilamani, P. (2002) *Proc. Annu. Conv. STAI*, **64**, 102–115.
48 Lewin, J. and Reimann, B.E.F. (1969) *Annu. Rev. Plant Physiol.*, **20**, 289–304.
49 Cheong, W.Y.Y., Heits, A., and De Ville, J. (1972) *Proc. Int. Soc. Sugar Cane Technol.*, **14**, 766–776.
50 Srinivasa Rao, Ch., Prasad, J.V.N.S., Vittal, K.P.R., Venkateswarlu, B., and Sharma, K.L. (2003) *Fert. News*, **48**, 105–114.
51 Tandon, H.L.S. (1987) *Micronutrient News*, **7**, 35.
52 Durai, R. (1997) *Coop. Sugar*, **29**, 102–104.
53 Kathiresan, G. and Balasubramanian, N. (1991) *Indian Sugar*, **41**, 319–324.
54 Manoharan, M.L., Duraisamy, K., and Vijayaraghavan, H. (1990) *Bharatiya Sugar*, **15**, 19–31.
55 Parameswaran, P. and Ramakrishnan, M.S. (1987) *Indian J. Sugarcane Technol.*, **4**, 113–118.
56 Ramakrishna Rao, S., Raju, J.S.N., Veerbhadra Rao, K., Padmanabhan, M., Sadanandachari, A., and Ramapandu, S. (1988) *Proc. Deccan Sugar Tech. Assoc.*, **38**, 55–64.
57 Page, R.E., Glanville, T.J., and Truong, P.N. (1986) The significance of trash retention trials in the Isis and Maryborough mill areas (Queensland) sugarcane. Paper presented at the Conference Australian Society for Sugar Cane Technologists, Townsville, Queensland, April 28–May 1.
58 Durai, R., Chinnaswami, K.N., and Kumarswamy, K. (1996) *Indian J. Agron.*, **41**, 468–471.
59 Bendigeri, A.V. and Pawar, M.W. (1997) *Bharatiya Sugar*, **22**, 23–40.
60 Li, Y. and Solomon, S. (2003) *Sugar Tech.*, **5**, 213–233.
61 Parthasarathy, S.V. (1972) *Sugarcane in India*, KCP Ltd., Madras, India, p. 804.
62 Nayak, N., Das, P.K., Mahapatra, S.S., and Jena, B.C. (1997) *Indian Sugar*, **47**, 265–269.
63 Srivastava, S.C., Johari, D.P., and Gill, P.S. (1988) *Manual of Sugarcane Production in India*, Indian Council of Agricultural Research, New Delhi, p. 194.
64 Smith, D. (1978) *Cane Sugar World*, Palmer Publications, New York, p. 240.
65 Singh, K. (1990) *Indian Sugar*, **40**, 537–539.
66 Pandey, U. (1964) *Proc. Bienn. Conf. S RDWI*, **5**, 340–344.

67 Rakkiyappan, P. (1982) Proceedings of the 4th Sugarcane Research and Development Workers Meeting (July 23–24) at Thiru Aroran Sugars Ltd., Vadapathimanaagalam, Tamil Nadu, India

68 Singh, G., Singh, O.P., Singh, R.S., Yadav, R.A., and Singh, B.B. (1991) *Bharatiya Sugar*, **16**, 27–30.

69 Patel, B., Shrivastava, P.K., Lad, A.N., and Raman, S. (2000) Proceedings of the 8th ICID International Drainage Workshop on Role of Drainage and Challenges in 21st Century, New Delhi, India, vol. I. 31 January–4 February, pp. 203–209.

70 Lal, C., Yadav, B.P., and Jaiswal, C.S. (2000) Proc. 8th ICID International Drainage Workshop on Role of Drainage and Challenges in 21st Century, New Delhi, India, vol. I. 31 January–4 February, pp. 277–288.

71 Singh, M., Ahuja, R.L., and Khanna, S.S. (1985) Soils of Haryana and their management, in *Soils of India and Their Management*, Fertilizer Association of India, New Delhi, pp. 130–148.

72 Reeve, R. and Fireman, M. (1967) Salt problems in relation to irrigation, in *Irrigation of Agricultural Lands*, American Society of Agronomy, USA, pp. 988–1011.

73 Sinha, M.K., Sarkar, A.K., Sahi, B.P., Singh, R.N., and Sahay, S. (1985) Soils of Bihar and their management, in *Soils of India and Their Management*, Fertilizer Association of India, New Delhi, pp. 46–71.

74 Yadav, J.S.P. (1987) Banzar mein hariyali, in *Ann Utpadan mein Atmanirbharata ka Yug: Indira Ji Ko ek Shrandhanjali*, Indian Council of Agricultural Research, New Delhi, India (in Hindi).

75 Raman, K.V., Sreenivasa Raju, A., and Krishnamoorthy, P. (1985) Soils of Andhra Pradesh and their management, in *Soils of India and Their Management*, Fertilizer Association of India, New Delhi, pp. 19–45.

76 Anonymous (1992) *BSES Bull.*, **40**, 10–11.

77 Ham, G.J., Cox, A.Z., and McMahon, G.G. (1997) Proceedings of the Conference of the Australian Society of Sugar Cane Technologists (Cairns, Queensland), 200–207.

78 Johnston, M.A. (1977) *Proc. Congr. S. Afr. Sugar Technol. Assoc.*, **51**, 42–46.

79 Fearon, C.G. (1996) Proceedings of the West Indies Sugar Technologists XXV Conference, Belize, April 18–21, 1994, 159–165.

80 Wang, P.L., Yang, P.C., Chang, Y.T., and Yao, T.P. (1989) *Rep. Taiwan Sugar Res. Inst.*, **124**, 13–21.

81 Li, S.-W. and Wang, P.-L. (1991) *Taiwan Sugar*, **38**, 8–10.

82 Wang, P.L., Cheng, Y.T., and Yao, T.P. (1990) *Rep. Taiwan Sugar Res. Inst.*, **127**, 11–25.

83 Bhan, V.M. (1963) *Indian J. Sugarcane Res. Dev.*, **8**, 62–64.

84 Kanzaria, M.V. and Patel, M.S. (1985) Soils of Gujarat and their management, in *Soils of India and Their Management*, Fertilizer Association of India, New Delhi, pp. 103–129.

85 Perur, N.G. and Mithyantha, M.S. (1985) Soils of Karnataka and their management, in *Soils of India and Their Management*, Fertilizer Association of India, New Delhi, pp. 177–207.

86 Patil, N.D. and Ghonsikar, C.P. (1985) Soil of Maharashtra and their management, in *Soils of India and Their Management*, Fertilizer Association of India, New Delhi, pp. 250–265.

87 Sundara, B. (1996) *Bharatiya Sugar*, **22**, 35–39.

88 Shannon, E. (1994) *BSES Bull.*, **46**, 10–11.

89 Zende, N.A. and Hapase, D.G. (1986) *Bahratiya Sugar*, **11**, 41–48.

90 Panje, R.R., Gill, P.S., and Alam, M. (1966) *Indian Sugar J.*, **10**, 1–8.

91 Feigin, A. (1985) *Plant Soil*, **89**, 285–299.

92 Rege, R.D. and Basu, J.K. (1948) *Sugarcane Research in Bombay State: 1932–1942*, Govt. Printing Press, Bombay, India.

93 Awad, A.S., Edwards, D.G., and Campbell, L.C. (1990) *Crop Sci.*, **30**, 123.

94 Nieman, R.H. and Clark, R.H. (1976) *Plant Physiol.*, **57**, 157–161.

95 Treeby, M.T. and van Steveninck, R.F.M. (1988) *Physiol. Plant.*, **73**, 317–322.

96 Hawaiian Sugar Planter's Association (1983) Annual Report of the Hawaiian

Sugar Planter's Association Experiment Station, Hawaii, USA.
97 Ashraf, M., Rahmatullah, A.R., Bhatti, A.S., Afzal, M., Sarwar, A., Maqsood, M.A., and Kanwal, S. (2010) *Pedosphere*, **20**, 153–162.
98 Potty, N.N., Vergheese, S.S., and Nair, S.S. (1985) *Indian Sugar*, **35**, 401–404.
99 Coale, F.J. (1993) *J. Am. Soc. Sugar Cane Technol.*, **13**, 87–94.
100 Nixon, D.J., Meyer, J.H., Mcarthur, D., and Shumann, A.W. (2003) Proceedings of the South African Sugar Technologists Association; Sugar Cane International 22 (1), 2004.
101 Mutert, E.W. (1997) Fertility management of tropical acid soils for high and sustainable sugar production in SE Asia. Paper presented at the Ist Asia-Pacific Sugar Conference, Philippines.
102 Taiwan Sugar Research Institute (1984) Annual Report of Taiwan Sugar Research Institute, Taiwan, p. 7.
103 Singh, O. (1993) *Indian Sugar*, **43**, 319–321.
104 Ulloa, M.F. and Anderson, D.L. (1991) Sugarcane cultivar response to calcium silicate slag on Everglades Histosols. Paper presented at the ASSCT Annual Meetings, New Orleans, LA.
105 Anonymous (1963) Annual Progress Report of the Research Work on Sugarcane Conducted in Uttar Pradesh at Shahjahanpur, India.
106 Panwar, B.S., Verma, R.S., and Srivastava, S.N.L. (1989) *Coop. Sugar*, **20**, 409–410.
107 Ricaud, R. and Arceneaux, A. (1986) *Proc. Int. Soc. Sugar Cane Technol.*, **19**, 18–24.
108 Louisiana State University (1987) Annual Progress Report of Sugarcane Research, Louisiana, Agricultural Experiment Station Louisiana State University, Louisiana, USA. Kanwar, R.S. and Kaur, H. (1977) *Proc. Int. Soc. Sugar Cane Technol.*, **16**, 1325–1331.
109 Peng, S.Y. and Twu, L.T. (1978) *Taiwan Sugar*, **25**, 8–17.
110 Yang, P.C. (1986) *Taiwan Sugar*, **33**, 17–25.
111 Shrivastava, A.K., Singh, K., Yadav, R.L., Kacker, N.K., and Singh, M. (1981) Proceedings of the National Seminar on Ratoon Management (IISR, Lucknow), I, pp. 100–108.
112 Chauhan, R.S., Verma, R.S., and Pathak, K.C. (1984) *Coop. Sugar*, **16**, 135–138.
113 Singh, O. (1988) *Indian Sugar*, **38**, 103–110.
114 Srivastava, A.K., Solomon, S., Rai, R.K., Jain, R., Singh, P., Kumar, R., Sawnani, A., and Shukla, S.P. (2011) X Agricultural Science Congress on Soil, Plant and Animal Health for Enhanced and Sustained Agricultural Productivity February 10–12, NBFGR, Lucknow, India (Abstracts, SFH-100), p. 106.
115 Yang, P.C. and Ho, F.W. (1980) *Proc. Int. Soc. Sugar Cane Technol.*, **17**, 711–724.
116 Sharma, H.K., Singh, O., and Kanwar, R.S. (1987) Proceedings of the Annual Convention of STAI, 50, pp. 81–86.
117 Kumar, A. and Pande, H.P. (1991) *Bharatiya Sugar*, **16**, 49–56.
118 Shukla, S.K., Yadav, R.L., Singh, P.N., and Singh., I. (2009) *Eur. J. Agron.*, **30**, 27–33.
119 Mathur, P.S. (1987) Agronomical practices for better ratoon production, in *Platinum Jubilee Souvenir*, vol. **I**, Sugarcane Breeding Institute, Coimbatore, India, pp. 135–139.
120 Shrivastava, A.K., Ghosh, A.K., and Agnihotri, V.P. (1992) *Sugar Cane Ratoons*, Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, pp. 182.
121 Singh, U.S. and Singh, L. (1967) *Indian Sugar*, **17**, 475–481.
122 Clements, H.F. (1965) *Proc. Int. Soc. Sugar Cane Technol.*, **12**, pp. 197–215.
123 Earle, F.S. (1928) *Sugarcane and Its Culture*, John Wiley & Sons Inc., New York, p. 355.
124 Ramana Rao, T.C., Sreenivasan, T.V., and Palanichami, K. (1985) Catalogue on Sugarcane Genetic Resources – II *Saccharum barberi*, Jeswiet, *Saccharum sinense*, Roxb. Amend. Jeswiet, *Saccharum robustum* Brandes et Jeswiet ex., *Saccharum edule* Hassk. Grassl. SBI, Coimbatore, India.
125 Sreenivasan, T.V. and Bhagyalakshmi, K.V. (2001) *Recently Released Sugarcane Varieties*, Sugarcane Breeding Institute, Coimbatore, India, p. 29.

126 Arceneaux, G., Coleman, R.E., and Hebert, L.P. (1951) *Proc. Int. Soc. Sugar Cane Technol.*, **7**, 116–127.

127 Irvine, J.E. (1978) *Proc. Int. Soc. Sugar Cane Technol.*, **16**, 147–156.

128 Brands, E.W. (1939) *Sugar Bull.*, **18**, 3–5.

129 Roach, B.T. and Maynard, E.O. (1975) *Sugarcane Breeder's Newslett.*, **35**, 43–49.

130 Dunckelman, P.H. and Breaux, R.D. (1972) *Proc. Int. Soc. Sugar Cane Technol.*, **14**, 233–239.

131 Moore, P.H. (1987) Breeding for stress tolerance, in *Sugarcane Improvement Through Breeding* (ed. D.J. Heinz), Elsevier, Amsterdam,, pp. 503–542.

132 Veremis, J.C., Tew, T.L., and Pan, Y.-B. (2003) Proceedings of the Annual Meeting of the American Society of Sugar Cane Technologists;*Sugar Cane Int.*, Nov./Dec. 2003, 28.

133 Sreenivasan, T.V. and Sreenivasan, J. (2000) *SBI Newslett.*, **19**, 1–2.

134 Machado, G.R., Jr. and Burnquist, W.L. (1986) *Variety Notes* (Fourth Revision), Copersucar Tech. Centre, Piracicaba, Sau Paulo, Brazil, p. 78.

135 Flores, S. (2003) *Sugar J.*, **66**, 23, 25.

136 Krishnamurthi, M. (1989) Sugarcane genetic resources and their utilization through conventional and unconventional approaches, in *Sugarcane Varietal Improvement* (eds K.M. Naidu, T.V. Sreenivasan, and M.N. Premchandran), SBI, Coimbatore, India, pp. 163–176.

137 Sreenivasan, T.V., Amalraj, V.A., and Jebadhas, A.W. (2001) *Catalogue on Sugarcane Genetic Resources IV. Erianthus Species*, Sugarcane Breeding Institute, Coimbatore, India, p. 98.

138 Shrivastava, A.K., Shahi, H.N., Kulshreshtha, N., Shukla, S.P., and Darash, R. (2001) International Plant Nutrition Colloquium Hannover, Germany XIV, July 27–August 3 (Abstract No. S1 A 218).

139 Srinivasan, T.V. (2004) *Sugar Tech.*, **6**, 107–111.

140 Jackson, P.A. (2005) *Field Crops Res.*, **92**, 277–290.

141 Misra, S.R. and Chaudhary, S.K. (2003) *Indian Sugar*, **53**, 19–25.

142 Tew, T.L. (1987) New varieties, Chapter 15, in *Sugarcane Improvement Through Breeding* (ed. D.J. Heinz), Springer, New York, pp. 559–594.

143 Epstein, E. and Rains, D.W. (1987) *Plant Soil*, **99**, 17–29.

144 Grover, A., Kapoor, A., Katiyal, S., Agrawal, M., Sahi, C., Jain, P., Kotak, S., Agrawal, S., and Dubey, H. (2001) *Proc. Indian Nat. Sci. Acad. (PINSA), B*, **67**, 189–214.

145 Wang, Z.Z., Zhang, S.Z., Peng, Y.B., and Li, Y.R. (2003) *Agric. Sci. China*, **12**, 19–26.

146 Luis, E.T., Carmen, M., Ochogavía, M.E., Hernández, I., Borrás, O., Rodríguez, R., Coll, Y., Arrieta, J.G., Banguela, A., Ramírez, R., and Hernández, L. (2009) *Biotechnol. Appl.*, **26**, 168–171.

147 Rodrigues, F.A., Laia, M.L., and Zingaretti, S.M. (2009) *Plant Sci.*, **176**, 286–302.

148 Chagas, R.M., Silveira, J.A.G., Ribeiro, R.V., Vitorello, V.A., and Carrer, H. (2008) *Pesticide Biochem. Physiol.*, **90**, 181–188.

149 Pedrozo, C.A., Jifon, J., Barbosa, H.P.M., Park, J.-W., Garcia, N.S., Jorge, A., and da Silva, J.A. (2010) Plant and Animal Genomes Conference, XVIII, January 9–13, San Diego, CA.

150 Gupta, V., Raghuvanshi, S., Gupta, A., Saini, N., Gaur, A., Khan, M.S., Gupta, R.S., Singh, J., Duttamajumder, S.K., Srivastava, S., Suman, A., Khurana, J.P., Kapur, R., and Tyagi, A.K. (2010) *Funct. Integr. Genomics*, **10**, 207–214.

151 Iskandar, H.M., Casu, R.E., Fletcher, A.T., Schmidt, S., Xu, J., Maclean, D.J., Manners, J.M., Ismond, K.P., Good, A.G., and Peacock, W.J. (2000) *J. Exp. Bot.*, **51**, 89–97.

152 Prabu, G., Kawar, P.G., Pagariya, M.C., and Prasad, D.T. (2011) *Plant Mol. Biol. Rep.*, **29**. doi: 10.1007/s11105-010-0230-0

153 Zhang, S.-Z., Yang, B.-P., Feng, C.-L., Chen, R.-K., Luo, J.-P., Cai, W.-W., and Liu, F.-H. (2006) *J. Int. Plant Biol.*, **48**, 453–459.

154 Molinari, H.B.C., Marur, C.J., Daros, E., Campos, M.K.F., Carvalho, J.F.R.P., Bespalhok Filho, J.C., Pereira, L.F.P., and

Vieira, L.G.E. (2007) *Physiol. Plant.*, **130**, 218–229.
155 Jangpromma, N., Kitthaisong, S., Lomthaisong, K., Daduang, S., Jaisil, P., and Thammasirirak, S. (2010) *Am. J. Biochem. Biotechnol.*, **6**, 89–102.
156 Rojas, C., Thiebaut, F., Almeida, K., Chabregas, S., Guimarães, A., Vicentini, R., Hemerly, A., and Ferreira, P. (2010) miRNA regulation during biotic and abiotic stress in sugarcane. Plant and Animal Genomes Conference, XVIII, January 9–13, San Diego, CA.
157 Drew, M.C. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **48**, 223–250.
158 Liao, C.-T. and Lin, C.-H. (2001) *Proc. Natl. Sci. Council, ROCB*, **25**, 148–157.
159 Dennis, E.S., Dolferus, R., Ellis, M., Rahman, M., Wu, Y., Hoeren, F.U., Grover, A., Ismond, K.P., Good, A.G., and Peacock, W.J. (2000) *J. Exp. Bot.*, **51**, 89–97.
160 Walker, J.C., Howard, E.A., Dennis, E.S., and Peacock, W.J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6624–6629.
161 Sachs, M.M., Freeling, M., and Okimoto, R. (1980) *Cell*, **20**, 761–767.
162 Urao, T., Noji, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1996) *Plant Cell*, **5**, 1529–1539.
163 Khan, M.S., Gupta, V., Singh, J., Duttamajumder, S.K., Chauhan, J.S., Kapur, R., and Tyagi, A.K. (2010) A snapshot of water-logging stress transcriptome of sugarcane. Plant and Animal Genome Conference, XVIII, January 9–13, San Diego, CA.
164 Harinarain, R., Guzhov, P., Yu, L., and Dolgikh, I.Y. (1996) *Biology Bull. Russian Acad. Sci.*, **23**, 336–345.
165 Tanvir, M.K., Ali, R., Akhtar, S., Sajid-ur-Rahman, and Chaudhary, B.A. (2001) *Pakistan Sugar J.*, **16**, 32–35.
166 Prammanee, S., and Pitakpolrat, P. (2001) *Proc. Int. Soc. Sugar Cane Technol.*, **24**, 644–645.
167 Patade, V.Y., Suprasanna, P., and Bapat, V.A. (2008) *Plant Growth Regul.*, **55**, 169–173.
168 Patade, V.Y., Bhargava, S., and Suprasanna, P. (2009) *Agric. Ecosyst. Environ.*, **134**, 24–28.
169 Wahid, A. and Ghazanfar, A. (2006) *J. Plant Physiol.*, **163**, 723–730.
170 Upadhyaya, A., Davis, T.D., Larsen, M.H., Walsen, R.H., and Sankhla, M. (1990) *Physiol. Plant.*, **79**, 78–84.
171 Kunst, L., Bowse, J., and Somerville, C.R. (1989) *Plant Physiol.*, **91**, 401–408.
172 Raison, J.K. (1986) Alterations in the physical properties and thermal response of membrane lipids: correlations with acclimation to chilly and high temperature, in *Frontiers of Membrane Research in Agriculture* (eds J.B. St John, E. Berlin, and P.C. Jackson), Rowmann and Allanheld, Totoma, NJ, pp. 383–401.
173 Schoffl, F., Prandl, R., and Reindl, A. (1999) Molecular responses to heat stress, in *Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants* (eds K. Shinozaki and K. Yamaguchi-Shinozaki), K.R.G. Landes Co., Austin, pp. 81–98.
174 Close, T.J. (1997) *Physiol. Plant.*, **100**, 291–296.
175 Qureshi, S.A., Mandramootoo, C.A., and Dodds, G.T. (2002) *Agric. Water Manage.*, **54**, 37–48.
176 Moisyadi, S. and Harrington, H.M. (1989) *Plant Physiol.*, **90**, 1156–1162.
177 Wahid, A. and Close, T.J. (2007) *Biologia Plant.*, **51**, 104–109.
178 Abrol, Y.P. and Ingram, K.T. (1996) Effects of higher day and night temperatures on growth and yields of some crop plants, in *Global Climate Change and Agricultural Production* (eds B. Fakhri and W. Sombroek), John Wiley & Sons Ltd., West Sussex, England.
179 Mahan, J.R., Burke, J.J., and Orzech, K.A. (1987) *Plant Physiol.*, **82**, 518–522.
180 Nogueira, F.T.S., de Rosa, V.E., Jr., Menossi, M., Ulian, E.C., and Arruda, P. (2003) *Plant Physiol.*, **132**, 1811–1824.
181 Pearce, R.S. (1999) *Plant Growth Regul.*, **29**, 47–76.
182 Shrivastava, A.K., Srivastava, A.K., Kulshreshtha, N., Srivastava, S., Pathak, A.D., Solomon, S., Srivastava, B.L., Srivastava, M.K., Rai, R.K., Singh, I., Singh, P., Suman, A., Gupta, P.S., Sawnani, A., Prajapati, C.P., Gaur, A., Saxena, V.K., and Mishra, A.K. (2005) *Sugar Cane Int.*, **23**, 3–11.

183 Inman-Bamber, N.G., Bonnett, G.D., Spillman, M.F., Hewitt, M.H., and Glassop, D. (2010) *Crop Pasture Sci.*, **61**, 111–121.

184 Du, Y.C. and Nose, A. (2002) *Photosynthetica*, **40**, 389–395.

185 Du, Y.C., Nose, A., and Wasano, K. (1999) *Plant Cell Environ.*, **22**, 317–324.

186 Jain, R., Shrivastava, A.K., Solomon, S., and Yadav, R.L. (2007) *Plant Growth Regul.*, **53**, 17–23.

187 Wood, R.A. (1990) *Fert. Res.*, **26**, 87–98.

188 Meyer, J.H. and Wood, R.A. (2001) *Proc. S. Afr. Sug. Technol. Assoc.*, **75**, 242–247.

189 Du Toit, J.L., Beater, B.E., and Maud, R.R. (1962) *Proc. Int. Soc. Sugar Cane Technol.*, **11**, 101–111.

190 Figueira, A., Kido, E.A., and Almeida, R.S. (2001) *Genet. Mol. Biol.*, **24**, 207–220.

191 Barry, G.A., Bloesch, P., Gardner, E.A., and Rayment, G.E. (1998) *Proc. Conf. Aust. Soc. Sugarcane Technol.*, **20**, 69–75.

192 Liu, W.C., Tzeng, J.S., and Li, S.W. (1994) *Taiwan Sugar*, **4**, 9–17.

193 Jain, R., Shrivastava, A.K., and Srivastava, S. (2004) *Sugar Cane Int.*, **22**, 23–27.

194 Jain, R., Srivastava, S., and Shrivastava, A.K. (2009) *Trop. Agric. (Trinidad)*, **86**, 128–133.

195 Jain, R., Srivastava, S., Solomon, S., Shrivastava, A.K., and Chandra, A. (2010) *Acta Physiol. Plant.*, **32**, 979–986.

196 Jain, R., Srivastava, S., and Madan, V.K. (2000) *Indian J. Plant Physiol.*, **5**, 228–231.

197 Srivastava, S. and Jain, R. (2011) *J. Environ. Biol.*, **32** (6), 759–763.

198 Srivastava, S. and Jain, R. (2010) *J. Environ. Biol.*, **31**, 809–812.

199 des Vignes, W. (1987) *J. Agric. Soc. Trin. & Tobago*, **87**, 63–67.

200 Deren, C.W., Cherry, R.H., and Snyder, G.H. (1992) Proceedings of the American Society of Sugar Cane Technologists, 22 (June 17–19). (Abstract: *Sugar Y. Azucar*, 87, 2, 1992).

201 Azevedo, R.A., Carvalho, R.F., Cia, M.C., and Gratão, P.L. (2011) *Tropical Plant Biol.* doi: 10.1007/s12042-011-9067-4

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Sorghum: Improvement of Abiotic Stress Tolerance

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Sorghum, the fifth most important cereal crop in the world, provides food, feed, fodder, fiber, and fuel. It is the second cereal crop and first C4 photosynthetic plant for which whole genome is sequenced. The importance of this crop will increase tremendously in future due to its better adaptability to abiotic stresses, which are expected to increase because of global climate change and diminishing fresh water supplies, coupled with increasing demand for food and bioenergy. The yield potential of sorghum is evident from the fact that production of sorghum has been maintained despite a steady decline in its area of cultivation over the past three decades. In fact, the true yield potential of sorghum has rarely been realized, as it is mainly grown in areas of low rainfall and resource-poor agronomic conditions. Owing to its ability to survive in water-limiting conditions, sorghum has majorly been studied for its drought resistance mechanism. The drought response in sorghum differs depending on the occurrence of stress during preflowering and postflowering. Postflowering response is associated with stay-green trait. Quantitative trait loci (QTL) for pre- and postflowering have been identified. However, the underlying genes that confer drought tolerance in sorghum have not been mapped. Moreover, other morphophysiological traits such as epicuticular wax content, osmotic adjustment, membrane stability, water use efficiency, or drought-related root traits that have been postulated to play a significant role in drought resistance in sorghum have been largely unexplored. Molecular genetic and physiological dissection of these traits will be of immense significance. Aluminum toxicity is a major problem in acidic soils. QTL and gene mapping approach led to the mapping of a Multidrug and Toxic Compound Extrusion (*MATE*) gene in sorghum. Later *MATE* family genes were identified as potential candidates that underlie aluminum tolerance QTL in maize. Since the rice, sorghum, and *Brachypodium distachyon* genome sequences are already available, and with impending maize genome sequence, there is an immense opportunity for comparative genetics and genomics to dissect abiotic stress tolerance mechanisms in cereals. This will accelerate the gene discovery among the cereal crops and will help improve other plant species as well. Thus, sorghum with its smaller genome, wide germplasm resource, well-studied genetics, C4 photosynthesis, and adaptability to

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harsh environments represents optimal amalgamation for omics approaches to decipher drought resistance mechanism.

36.1 Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop in the world after wheat, rice, maize, and barley. Known for its ability to survive harsh environments with prolonged drought period, sorghum is grown in arid and semiarid areas of the world. It is a staple food in parts of Africa and Asia and a major feed crop in the United States, Mexico, Australia, and South Africa. It has extensive variability such as grain sorghum, forage sorghum, and sweet stalk sorghum that provides food, feed, fodder, fiber, and fuel. Sorghum is produced by about 104 countries in the world. In 2009, Sorghum was grown on 43.74 million ha of land worldwide with a yield of 14 198 Hg ha^{-1} (http://Faostat.fao.org/; December 20, 2009). Average area under sorghum cultivation in Asia has declined from 26.19 million ha in the 1960s to 10.58 million ha in 2008. However, yield increased from 6935 Hg ha^{-1} in the 1960s to 10 377 Hg ha^{-1} in the late 2000s (http://Faostat.fao.org/ ; December 22, 2009). The yield potential of sorghum is evident from the fact that production of sorghum has been maintained despite a steady decline in its area of cultivation. In fact, the true yield potential of sorghum has rarely been realized, as it is grown mainly in areas of low rainfall and resource-poor agronomic conditions. Its ability to yield under such agronomic and adverse climate conditions is a proof of concept that sorghum is the crop of the future.

In the changing global scenario, the world population is expected to rise from present 6.6 billion to 8.7–11.3 billion in 2050 [1]. The global demand for cereal production will also increase by 60% [2]. This task is challenging as the yield potential of cereal crops has reached its plateau, and there is reduction in cultivable land and availability of fresh water for irrigation. These problems are further exacerbated by global climate change-associated increase in the frequency of heat stress, droughts, and floods that negatively affect crop yields [3]. Ability of crops to adapt and yield under such harsh environment will be crucial in determining the sustainability of food production in days to come. This will require a combination of adaptive agricultural strategy that includes new management and agronomic practices and further improvement in the genetic potential of productivity and abiotic stress resistance of crops. This also implies that lessons need be learned from plants that show high adaptability and tolerance to abiotic stresses.

Sorghum belonging to genus *Poaceae* and subfamily Panicoideae shares the tribe Andropogoneae with other major crops such as maize, sugarcane, and millets. The Andropogoneae species are native to tropical and subtropical climates, and are characterized by C4 photosynthesis, high rates of carbon fixation, high water and nutrient use efficiency, high biomass productivity, adaptation to diverse

environments, and have both annual and perennial life cycles. However, many of these species are polyploids with large complex genomes. Sorghum, besides having all the advantageous characteristics, has a diploid genome that is already sequenced [4]. Moreover, with its well-studied genetics, wide germplasm resource, lower level of gene duplication compared to other tropical cereals, and amicability for genetic transformation, sorghum can be an ideal system especially for grasses and plant genomics research as a whole.

36.2 Abiotic Stress Tolerance

Abiotic stresses limit the growth and productivity of crop plants to variable degrees depending on the time of onset, duration, and intensity of stress. It has been estimated that crops attain only about 25% of their potential yield because of the detrimental effects of environmental stresses [5]. During the second half of twentieth century, increase in crop productivity by plant breeding efforts kept in pace with the food demand of the increasing world population. This was achieved mainly by breeding programs aimed at increasing yield potential and disease resistance. However, the progress in breeding for abiotic stresses has been very slow as, first, the mechanism of abiotic stress tolerance was poorly understood and, second, the breeding in the past 50 years was more yield oriented [6]. Since the relative rate of yield increase for major crops such as rice and wheat is declining [7], there is a need to adopt and intensify the physiological trait-based molecular breeding approach for breeding abiotic stress-tolerant crops [8]. Physiological breeding, also known as analytical breeding, refers to selection for secondary traits that are associated with higher yield under optimal and/or abiotic stress environments [6]. On the basis of the physiological traits that contribute to yield in soil moisture-deficit environments, a general model for drought adaptation of wheat was proposed [8]. The model describes four main groups of traits relating to (i) preanthesis growth, rapid ground cover to shade the soil to prevent evaporation, and high assimilation capacity between jointing and lag phase, to permit accumulation of stem carbohydrates; (ii) high rooting depth and/or intensity to access water that would be expressed by a relatively cool canopy or favorable expression of water relation traits; (iii) water use efficiency (WUE), photosynthesis associated with refixation of respiratory CO_2; and (iv) photoprotection including energy dissipation, antioxidant systems, and anatomic traits such as leaf wax [8]. Though these traits have been proposed for wheat *per se*, it may apply to any crop improvement program aimed at drought tolerance. Yet not all the crops will have the best amalgamation of these traits. Sorghum with its stay-green trait, deep rooting system, better WUE, C4 photosynthesis, and high epicuticular wax (EW) represents one good system to study physiological traits related to drought tolerance. However, genetic and molecular analyses of these traits are in its infancy. The chapter describes the progress in abiotic stress tolerance research and prospects for genetic improvement of sorghum. It includes the physiological trait-based studies conducted in

sorghum in relation to drought, followed by cold, salt, and aluminum tolerance and the genetic and genomic resources for further progress in crop improvement in sorghum.

36.2.1 Drought Tolerance

Drought stress is one of the most critical stress affecting plants. Drought can be defined in multiple ways, be it meteorological, hydrological, or socioeconomical context. When drought is defined in relation to crops or agriculture, it refers to shortage of water in the root zone that reduces yield [9]. When a genotype yields higher than another genotype under severe drought, it is ranked relatively more drought tolerant.

Plants deal with stress in three different ways, namely, escape, dehydration avoidance, and dehydration tolerance. Drought escape is defined as the ability of a plant to complete its life cycle before severe soil and plant water deficit develops. Escape mechanism involves rapid phenological development (early flowering and early maturity) and developmental plasticity (variation in duration of growth period depending on the extent of water deficit). Dehydration avoidance is defined as the ability of plants to sustain high plant water status or cellular hydration under drought conditions. Crop plants avoid dehydration by enhanced capture of soil moisture by efficient root system and osmotic adjustment (OA), by limited crop water loss from transpiration and other nonstomatal pathways such as through the plant cuticle, reduced absorption of radiation by radiation reflection, and leaf rolling/folding or drying. Dehydration tolerance is defined as the capacity to sustain or conserve plant function even in relatively low tissue water potential. Cellular water deficit stress tolerance in plants depends on modification of metabolism, production of organic compatible solutes (proline, sugars, polyols, betaine, etc.), and expression of genes involved in membrane integrity, cellular homeostasis (ionic-, osmotic-, and metabolic homeostasis), stress damage control, and repair.

Traits associated with avoidance and tolerance can be constitutive (intrinsic traits that express constitutively) or adaptive (traits that express in response to stress). Depending on the occurrence of stress at vegetative or reproductive stage, sorghum exhibits preflowering and postflowering stress response, respectively. These two responses are apparently controlled by different genetic mechanisms [10]. Preflowering stress affects biomass, panicle size, grain number, and grain yield [11], while postanthesis drought leads to premature leaf and stem senescence, lodging, and reduced seed size [12]. Postanthesis drought also increases susceptibility of plants to biotic stresses such as charcoal rot (*Macrophomina phaseolina* (Tassi) Goidanich) and fusarium stalk rot (*Fusarium moniliforme* J. Sheld.) [12].

For preflowering drought tolerance, six distinct genomic regions were identified in sorghum recombinant inbred lines (RILs) derived from the cross between Tx 7078 (preflowering-tolerant, postflowering-susceptible) and B35 (preflowering-susceptible, postflowering-tolerant) genotypes [13]. These loci accounted for approximately 40% of the total phenotypic variation in yield under preflowering drought and were

detectable across a range of environments. Kebede *et al.* [14] identified four quantitative trait loci (QTL) associated with preflowering drought tolerance in sorghum from RILs derived from the cross, SC 56 × Tx 7000. The major QTL influencing preflowering drought stress tolerance accounted for 15 and 37.7% of the phenotypic variance under two different environments, suggesting a strong G × E interaction at this loci.

36.2.1.1 **Stay Green**

Postflowering drought response is associated with stay-green trait in sorghum. Stay green is basically retention of green leaf area at maturity (GLAM). Maintenance of stay-green trait during grain-filling stage under soil moisture-deficit stress condition constitutes an important component of drought tolerance [15]. The stay-green phenotype has been classified into five types [16]. In type A stay green, the initiation of senescence is delayed, but proceeds at the same rate as the wild type. Type B stay green initiates senescence at the same time as the wild type, but senescence proceeds at slower rate. The above two types are regarded as functional stay green as retention of greenness is associated with extended photosynthetic activity during grain filling. On the other hand, type C or "cosmetic" stay green retains chlorophyll almost indefinitely; however, the photosynthetic rate declines. Type D stay green is the greenness retained after the leaf death by abrupt freezing or drying. Finally, type E stay green contains higher chlorophyll content to begin with, but follows senescence at normal time and rate.

Functional stay green can be of immense importance as it has been correlated with higher grain filling and increased yield under postanthesis drought [12]. Moreover, there is no yield penalty associated with stay green under nondrought conditions [12]. Stay green has also been associated with higher leaf nitrogen content [17, 18], reduced lodging [12], lower susceptibility to charcoal rot [19], and higher levels of stem carbohydrates both during and after grain filling [12]. Thus, stay green contributes to various aspects of crop improvement and hence is a valuable trait for crops like sorghum where primary harvest can be grain, forage, juice, and/or fodder.

In sorghum, different stay-green sources are available that include B35 (BTx 642), SC 56, E36-1, and KS19 [14, 20–22]. In breeding, B 35 and KS 19 are the two main sources used for stay green [20]. These two genotypes represent two different types of functional stay green: B 35-derived lines have a greater leaf area at flowering and a normal rate of leaf senescence, whereas KS 19-derived lines have a smaller leaf area at flowering and a slower rate of leaf senescence [12]. Although the ability of leaves to delay senescence has a genetic basis in sorghum, the expression of the character is strongly influenced by environmental factors [23]. The selection for trait depends upon the occurrence of a prolonged period of drought stress during the grain-filling period to accelerate normal leaf senescence. Genetic studies also showed that stay-green trait is governed by genes that act at varied levels of dominance or additive effects. For instance, the inheritance of the onset of senescence was additive, but a slow senescence rate was found to be dominant over a fast rate [23, 24]. Furthermore, the three components of stay-green trait, namely, green leaf area at flowering, time of

Table 36.1 Summary of studies related to identification of QTL for stay-green trait in sorghum.

Stay-green parent	Nonstay-green parent	Experimental location	Population	Markers used	Reference
B35	Tx 7078	Mexico USA	RIL	RAPD RFLP	[25]
B35	Tx 430	USA	RIL	RFLP	[26]
B35	Tx 7000	USA	RIL	RFLP	[27]
B35	Tx 7000	USA	RIL	RAPD RFLP SSR	[28]
QL41	QL39	Australia	RIL	RFLP SSR	[29]
SC56	Tx 7000	USA	RIL	RFLP	[14]
E36-1	IS9830	India	RIL	AFLP	[21]
E36-1	N13			RAPD RFLPSSR	

onset of senescence, and subsequent rate of senescence also appear to be inherited independently [12, 23].

Several studies have mapped QTL contributing to the stay-green trait (Table 36.1). Most of these studies used B35 or derivatives of B35 as the stay-green source [25–29]. These studies led to identification of four major QTL, namely, *Stg1*, *Stg2*, *Stg3*, and *Stg4*. QTL *Stg1* and *Stg2* are located on LG-03, *Stg3* on LG-02, and *Stg4* on LG-05, and account for 20, 30, 16, and 10% of the phenotypic variance, respectively [11, 27]. Among these, *Stg2* was found to be the most important QTL, followed by *Stg1*, *Stg3*, and *Stg4* [27]. *Stg2* was consistent in all the environments, in different genetic backgrounds, and explained the highest percentage of phenotypic variation (~30%) in three different studies [26–28]. The near-isogenic lines (NILs) derived from the cross between B35 and RTx 7000 were evaluated under drought conditions at postflowering stage for their expression of stay-green phenotype. Physiological analysis of four NILs containing individual QTL, namely, *Stg1*, *Stg2*, *Stg3*, or *Stg4*, showed that B35 alleles in each of these loci could contribute to the stay-green phenotype. It was found that NILs having the genomic DNA of B35 spanning the region of the *Stg2* were performing better than NILs having other QTL. NILs with *Stg2* were showing higher GLAM and SPAD values and lesser rate of leaf senescence over others [30].

Stay-green expression is affected by the degree of stress during grain filling, and other factors such as flowering time and sink strength. It can be better manipulated using a marker-assisted breeding approach [31]. Therefore, efforts have also been initiated to transfer this trait through marker-assisted backcrossing (MABC) into elite cultivars and study their expression in different background [22, 31]. However, precision of marker-assisted breeding depends on how tightly the markers are linked to the genes or QTLs involved. Therefore, fine mapping of stay-green QTL still remains a prerequisite. Fine mapping of QTL can be achieved by increasing marker

Table 36.2 List of selected genes in the corresponding *Stg2* QTL region of BTx623 (http://www.phytozome.net).

Marker name	Position in chromosome 3 (bases)	Candidate genes	Predicted function
CSU58	54 878 005	—	—
RZ323	55 631 111	Sb03g027940	Similar to membrane-associated salt-inducible protein-like
		Sb03g028070	Similar to protein phosphatase 2C
		Sb03g028210	Similar to proline transport protein 2-like
		Sb03g028240	Similar to probable indole-3-acetic acid-amido synthetase GH3.5
		Sb03g028470	Similar to heat shock factor RHSF13
UMC63	57 218 551	Sb03g029190	Similar to carbonic anhydrase, chloroplast precursor
Xtxp002	57 539 612	Sb03g029570	Similar to malate dehydrogenase
		Sb03g029740	Similar to leaf senescence protein-like
		Sb03g029760	Similar to leaf senescence protein-like
		Sb03g030110	Similar to pyruvate kinase
WG889	58 956 759	—	—
Xtxp 114	60 794 047	—	—

density within the chromosomal region of interest and/or increasing the number of segregating population for which phenotypic information can be obtained. With available genome sequence and genomics tools, increasing the marker density appears to be more straightforward approach. Many sequence-based markers (namely, SNPs) can be made. That will further help in fine-mapping the QTL. Simultaneously, integrated genomic approaches can be used for deciphering the stay-green trait in sorghum. For instance, location of *Stg2* on available physical map of sorghum between markers RZ323 and WG889 [27, 28] in third chromosome of sorghum consists of more than 200 genes (Table 36.2). Some of these genes are predicted to function in important physiological processes such as photosynthesis, leaf senescence, and abiotic stress response (Table 36.2) that may contribute to the stay-green phenotype. Expression profile of these putative candidate genes can be correlated with the stay-green trait. This will narrow down the search for genes responsible for the trait.

On the basis of *in silico* comparative genome analysis, a few markers have already been developed in *Stg* QTL of sorghum [32]. Moreover, QTL for stay-green trait in wheat and rice have also been identified [33, 34]. Hence, comparative studies can be used to expedite the process of identifying genes responsible for stay green not only in sorghum but also in other cereals.

In addition to functional stay-green genotypes, stay-green mutants are also reported in many different species including rice [35], soybean [36], tomato [37], *Phaseolus vulgaris* [38], pepper [39], *Festuca pratensis* [40], and so on. The impetus on

identifying gene responsible for stay-green phenotype in these mutants started with the finding of a single recessive nuclear gene, *sgr (t)*, from a rice mutant [35]. Later, two research groups reported that s*gr* is a senescence-associated gene encoding a novel chloroplast protein. It was shown that the stay greenness of the *sgr* mutant was associated with a failure in the destabilization of the light-harvesting chlorophyll binding protein (LHCP) complexes of the thylakoid membranes, which is a prerequisite event for the degradation of chlorophyll and LHCPs during senescence [41, 42]. This was followed by identification of orthologous genes responsible for the stay-green character in other mutants that include Mendel's *green cotyledon* mutant in pea, *green-flesh* (*gf*) and *chlorophyll retainer* (*cl*) mutations of tomato and pepper, respectively [43, 44]. Though *sgr* has been associated with type C or cosmetic stay-green phenotype, yet it gives an insight into the mechanism of dismantling of photosynthetic chlorophyll–apoprotein complexes. It also implies that if found orthologous in nature, identification of functional stay-green genes in one species will speed up their elucidation in other cereal crops as well.

36.2.1.2 **Epicuticular Wax**

Epicuticular wax forms an outer visible glaucous coating on many crop plants called as waxy bloom or bloom. The accumulation of wax varies greatly depending on species, organ, stage of development, and environmental conditions. EW is highly diverse in composition and structure. Its hydrophobic composition and distribution on many aerial organs of plants has been considered a potentially useful trait and has been associated with resistance to many diverse environmental stresses including drought, insect, and disease resistance [45–47].

Sorghum is distinct from other cereal crops due to its ability to produce profuse amount of epicuticular wax (EW or bloom) that is deposited on abaxial leaf blade and sheath and culms, especially during preflowering and at maturity stages. The wax composition of sorghum leaf sheath shows highest (96%) level of free fatty acids with chain length varying from 16 to 33 carbons, of which C28 and C30 represent 78 and 20% of the constituents, respectively [48]. Moreover, sorghum as a species has been reported to produce one of the highest amounts of leaf EW among cereal crops. Burow *et al*. [49] reported that on a per unit leaf area basis, sorghum produces an average of 1.9 mg dm^{-1}, while the reported value for rice (*Oryza sativa* L.) is 0.05 mg dm^{-1} [50]. Similarly, on per unit weight basis, sorghum produces approximately 52.7 mg g^{-1} wax, which is 3-fold higher than that of maize (17.0 mg g^{-1}) [51] and 1.5–2-fold higher than that of durum wheat (25–35.7 mg g^{-1}) [52].

The most common plant waxes are very long-chain aliphatic molecules, of mainly 16–34 carbons in length, that occur as free fatty acids, aldehydes, primary alcohols, alkanes, and esters [53]. However, there exists a difference in the biosynthetic pathway depending on the carbon length. Synthesis of fatty acids with 16 carbons or less, acyl chains is activated by a soluble plastidic acyl carrier protein (ACP) and elongated by a fatty acid synthase (FAS) complex that condenses acetyl groups from malonyl-ACP to growing acyl-ACP chains [54]. Acyl-CoA of 16C or 18C chain length is exported from plastid into endosplasmic reticulum for long-chain acyl-CoA

synthesis. Acyl chains that serve as direct wax precursors are activated by coenzyme A (CoA) and elongated by membrane-associated enzyme complexes called fatty acid elongases [55]. Elongases use malonyl-CoA as the two carbon donors instead of malonyl-ACP. Once synthesized, the very long acyl-CoA chains are catalyzed by other enzymatic reactions and form free acids, esters, aldehydes, and alkanes that constitute the EW [48]. Thus, being involved in early steps in the wax metabolic pathway, acyl-CoA elongases may serve as rate limiting and highly regulated reactions, and hence plays a pivotal role in overall plant cuticular wax biosynthesis [56]. Genetic analysis of *Arabidopsis* mutant led to the identification of two enzymes of FAE complex, namely, *ECERIFERUM6* (*CER6*, β-ketoacyl-CoA synthase) and *CER10* (enoyl-CoA reductase). CER4, fatty acyl-CoA reductase, synthesizes primary alcohol from very long-chain fatty acids. The WSD1, wax synthase/fatty acyl-CoA: fatty alcohol acyltransferase synthesizes wax esters. Most of this information on biosynthetic pathway of plant EW has been built on genetic analysis of *Arabidopsis* mutants [57]. Though a few genes have now been isolated and characterized in rice and maize [47, 58], none of the genes has been characterized at molecular level in sorghum. However, there have been some detailed genetic studies on chemically induced mutants in sorghum [48, 59, 60]. These mutants were designated *bloomless* (*bm*), which completely lacked visible waxes on sheath surfaces and *sparse-bloom* (*h*), those with reduced visible sheath waxes [59]. *bm* and *h* wax mutants produced significantly low wax load compared to wild type. It was found that all the 12 *bm* mutants had a reduction in the amount of C28 and C30 fatty acids that resulted in the reduction of total wax load relative to wild type [48]. On the basis of composition analysis, it was suggested that these sorghum mutants may have lesions that affect either C26 acyl-CoA elongation or acyl-CoA thioesterases. The molecular identity of these mutant loci still remains unknown. These wax mutants can be exploited for elucidating genes involved in the biosynthesis of the very long-chain fatty acids. Recently, a mapping population developed from a cross between BTx623 (wild type with profuse wax) and KFS2021 (a mutant with greatly reduced wax) was used for molecular mapping and characterization of a locus associated with production of profuse wax *BLOOM-CUTICLE* (*BLMC*) in sorghum [61]. The locus mapped to the terminal end of sorghum chromosome 10 was delimited to as small as 0.7 cM region. The analysis of putative genes in the *BLMC* region revealed the presence of an acyl CoA oxidase (a gene involved in lipid and wax biosynthesis) and seven other putative transcripts, among others [61]. Next to stomata, water loss from plants occurs through its cuticle. High cuticular wax minimizes nonstomatal water loss from the plants. The bloomless F_2 progenies of the cross showed a significant negative correlation between leaf epicuticular wax load with epidermal permeability and night-time conductance, suggesting that epicuticular wax may enhance water use efficiency of sorghum by regulating night-time water loss [49]. In addition to disrupting the epicuticular wax production, *blmc* mutation also reduced culm and leaf cuticle, and increased plant death rate in the field at anthesis [61]. This phenotype was similar to *bm22* mutant reported by Jenks *et al.* [60]. The *bm 22* mutant reduced both epicuticular wax and cuticle deposition that in turn was associated with increased epidermal conductance to water vapor and increased susceptibility to the

funga1 pathogen *Exserohilum turcicum* [60]. Thus, there seems to be a link between the pathways of epicuticular wax synthesis and cuticle formation. Cutin synthetic enzymes use C16 and C18 acyl-CoA pools as precursors, potentially the same precursors used in wax synthesis. As cuticle is involved in several different functions including inhibition of uncontrolled permeation of water, solutes, and gases, and protection from phytopathogens and so on, identification and characterization of gene affecting both cuticle and epicuticular wax can be of significant importance for both biotic and abiotic stress tolerance of sorghum.

36.2.1.3 **Osmotic Adjustment**

Osmotic adjustment and antioxidant capacity are the two traits that have been associated with drought tolerance mechanisms. OA refers to the lowering of osmotic potential due to the net accumulation of compatible solutes in response to water deficits. These compatible solutes may be various amino acids (e.g., proline), sugars (e.g., sucrose and fructans), polyols (e.g., mannitol and pinitol), quaternary amines (e.g., glycine betaine), ions (e.g., potassium), and organic acids (e.g., malate and citrate) [62]. There is a wide variation in OA in crop plants, and the solutes accumulated also differ by plant species [63]. Osmotic adjustment is an inherited trait and has been associated with sustained yield in water-limiting conditions in many crop plants [15]. In sorghum, two independent major genes (*OA1* and *OA2*), with some minor effects, have been reported to control the inheritance of OA in sorghum [64].

Glycine-betaine (GB) is an important osmoprotectant and its role in abiotic stress tolerance is demonstrated in several plant species. GB stabilizes the quaternary structure of proteins, stabilizes highly ordered state of membranes, and reduces lipid peroxidation under stress [65]. Betaine aldehyde dehydrogenase and choline monooxygenase catalyze the synthesis of GB in a two-step oxidation of choline via the intermediate betaine aldehyde. In sorghum, expression of *BADH1* and *BADH15*, encoding betaine aldehyde dehydrogenase, was found to be induced by water deficit and their induced expression coincided with GB accumulation [66]. Among cereal crops, maize and sorghum synthesize GB, while rice does not [67]. Moreover within maize and sorghum, there are certain genotypes that do not accumulate GB [68, 69]. To study the GB accumulation in sorghum, near-isogenic lines (NILs) that differ in their ability to accumulate GB were analyzed [69]. Labeling studies in sorghum demonstrated that the deficiency in GB accumulation was at the choline oxidation step [69]. However, a recent study suggests that low GB accumulation may not be due to the absence of choline monooxygenase; rather, it may be due to the nonavailability of substrate or lack of choline transporter [70]. Thus, mechanism of GB synthesis and accumulation in these lines of sorghum and maize still remains an enigma.

Besides GB, other solutes such as proline, K^+, sugars, Cl^-, and P, were also reported to contribute to osmotic adjustment in sorghum [66, 71]. Since phenotyping for OA trait is difficult due to methodological constrains of OA evaluation, it will be important to map OA QTL for different solutes, which can be transferred by marker-assisted selection (MAS) or transgenic approach to incorporate this trait for improvement of OA and osmoprotection in sorghum.

36.2.2
Cold Tolerance

Sorghum being native to tropical and subtropical regions of Africa [72] is well adapted to warm growing conditions. Cool temperatures during the early growing season are, therefore, a major limitation to growing sorghum in temperate areas. The development of sorghum cultivars with improved early-season cold tolerance would allow expansion of sorghum to these more northerly latitudes and would also allow for earlier planting in areas where it is being grown [73]. Moreover, improved emergence and early-season vigor would enable better stand establishment and protect against loss of seedlings during unexpected cold periods that are likely to become frequent due to climate change scenario. Though most of the available sorghum germplasm is of tropical origin, some of the sorghum landraces from temperate regions of China, referred to as "kaoliang," exhibit higher seedling emergence and greater seedling vigor under cold conditions than most sorghum cultivars [74–76]. However, these races lack desirable agronomic characteristics. Hence, efforts are being made to introgress desirable genes from Chinese landraces into elite lines by marker-assisted selection. A population developed from a cross between Chinese landrace "Shan Qui Red" (SQR, cold tolerant) and SRN39 (cold sensitive) was employed for QTL analysis of early-season cold performance in sorghum [77]. Two QTL, one on linkage group SBI-03 and the second on group SBI-07, for germination under cold and optimal temperatures were identified. Another QTL located on linkage group SBI-01 showed strong association with seedling emergence and seedling vigor scores under early and late field planting. The three QTL were validated across two populations [78]. Hence, these can be useful for marker-assisted breeding to improve early-season performance in sorghum.

36.2.3
Salt Tolerance

Salinity is one of the major abiotic stresses that adversely affect crop productivity and quality [5]. Saline soil is characterized by toxic levels of chlorides and sulphates of sodium. The problem of soil salinity exists in both irrigated and dry areas. In irrigated areas, poor quality of water or improper drainage or entry of seawater in coastal areas contributes to salinity. In arid and semiarid regions, it is the high evaporation and insufficient leaching of ions due to inadequate rainfall that leads to high salt accumulation in root zones [79]. Salinity restricts plant growth due to nutritional constrains, ion toxicity, and osmotic stress. Though sorghum has been characterized as moderately tolerant, it is more tolerant than maize [80, 81]. Moreover, its better suitability in arid and semiarid regions also makes it a suitable target for improvement in salt tolerance. The mechanism of salt tolerance has been studied in detail (reviewed in Refs [79, 82]), yet the complex genetic mechanism is a big hurdle to improvement of salt tolerance. In sorghum, diallel analysis, based on relative root length in salt-treated and control plants, showed both additive and dominance effects of NaCl [83]. A large genotypic variation for tolerance to salinity in sorghum has been reported [83–86]; however, no detailed studies have been carried out. Therefore, there is a need to explore this area of stress tolerance in sorghum.

36.2.4
Aluminum Tolerance

Aluminum (Al) is a light metal that makes up 7% of the Earth's crust and is the third most abundant element after oxygen and silicon [87]. Most of this Al occurs in the form of harmless oxides and aluminosilicates with only small amounts present in soluble forms in the rhizosphere. However, under acidic condition of soil (pH < 5), Al is solubilized into the phtotoxic trivalent cation, Al^{3+}. Aluminum toxicity primarily affects the root growth resulting in limited absorption of water and mineral nutrients [88], leading to a significant reduction in the quality of the grains on acid soils [89]. Acidic soil accounts for up to 50% of the world's potentially arable soils [90], of which larger part comes from tropic and subtropic areas of developing countries. A significant variation in Al tolerance is reported within some species [89]. In barley, Al tolerance appears to be monogenic [91], while in rice it is a quantitative trait [92]. Al tolerance is either simply inherited as single dominant gene in some genotypes of wheat or involves action of more than one gene in other genotypes [93–96].

Plants have evolved two physiological mechanisms to resist the effect of Al toxicity in acidic soils: exclusion of Al from the root apex and chelation mechanism. Exclusion mechanism is based on the external detoxification of Al, which protects the root apex against Al penetration. This is achieved by the secretion of organic acids from the root apex to the rhizosphere that modifies the pH and chelates the toxic Al^{3+} [97]. Chelation mechanism works on compartmentalization of aluminum ions by specific proteins, short-chain organic acids, phenolic compounds, and tannins that can bind and form complexes with Al^{3+}. These complexes are subsequently compartmentalized in the vacuole, thus reducing Al toxicity [98–100]. Among the two mechanisms, Al-activated exudation of organic acid – anions – from root apices is the best documented and characterized plant Al tolerance mechanism [87]. The exudation of organic acid may be species specific, such as malate from Al-tolerant cultivars of wheat [101], citrate from Al-tolerant cultivars of maize [102] and soybean [103], and oxalate from buckwheat [104] and taro [105]. However, some species such as *Secale cereale* (rye) may show exudation of both malate and citrate [106].

Transport of these organic acids occurs via anionic channels, the opening of which may be activated by Al. The first such transporter *ALMT1* (aluminum-activated malate transporter 1), responsible for malate efflux, was identified in wheat [107]. *ALMT1* represented a new family of membrane proteins and mapped to chromosome 4DL, corresponding to Alt_{BH}, a major aluminum tolerance locus in wheat and other members of the Triticeae tribe [108]. A new thrust came in to the Al tolerance research when Magalhaes *et al.* [109] reported a single locus, Alt_{SB}, which accounted for 80% of the Al tolerance phenotype in sorghum mapping population. Interestingly, the locus Alt_{SB} mapped on the sorghum chromosome 3, which is not homologous to the Triticeae group 4 chromosomes. Comparative mapping studies indicated that a major Al tolerance QTL on rice chromosome 1 might be orthologous to Alt_{SB}, whereas another QTL on chromosome 3 is likely to correspond to the Triticeae group 4 Al tolerance locus [109]. Therefore, it appeared that in rice that is one of the most Al-tolerant grasses [92, 110], the quantitative inheritance of Al tolerance may be a result of

two major QTL, which act as two independent and distinct major Al tolerance genes in Andropogoneae and Triticeae [109]. As Alt_{SB} appeared to be distinct from Alt_{BH}, positional cloning of Alt_{SB} was taken up that led to the identification of the gene encoding aluminum-activated citrate transporter, a member of the multidrug and toxic compound extrusion (*MATE*) family from sorghum [111]. Transgenic expression of *SbMATE* gene conferred Al tolerance in both *Arabidopsis* and wheat [111]. Simultaneously, in the same year another MATE protein, *HvAACT1*, an Al-activated citrate transporter that confers Al tolerance to barley, was reported [112]. MATE proteins are members of a large and complex family of transporters; functional members of this family were found first in prokaryotic organisms and later in eukaryotic organisms and are generally involved in the efflux of small organic solutes. Their identification in sorghum and barley subsequently led to the identification of several other plant MATE members that were implicated in citrate transport. These include *OsFRDL1* from rice [113], *AtMATE* from *Arabidopsis* [114], and *ZmMATE1* in maize [115], and a *MATE* gene implicated in citrate efflux has also been reported from wheat [116]. Though overall studies indicate that Al tolerance in plants is predominantly contributed by orthologous series of at least two major loci, detection of additional QTL or genes in the genomes of maize [117], rice [92], oat [118], and rye [119] indicates that these Al tolerance genes may also play a role in Al tolerance of plants.

In sorghum, Al tolerance appears to be a function of both allelic heterogeneity and nonallelic heterogeneity [120]. A wide range of phenotypic variation for Al tolerance was found, which was attributed to multiple alleles of Alt_{SB}. Even the two most tolerant sorghum cultivars, SC283 and SC566, which were found to rely on Alt_{SB} for their tolerance [109], showed a distinct phenotype, SC566, being significantly more tolerant than SC283 indicating that the SC566 allele is stronger than the SC283 allele [120]. As the correlation between *SbMATE* expression and Al tolerance in a panel having allelic diversity at the Alt_{SB} locus was highly significant [111], it was suggested that these allelic effects in part may be regulatory in nature. Moreover, transgressive segregation was also observed in a highly Al-tolerant breeding line, indicating the role of additive or codominant effects in sorghum Al tolerance [120]. Though identification of these nonorthologous and additive aluminum tolerance genes remains to be explored, the major gene Alt_{SB} from sorghum has been instrumental in revealing a new mechanism of aluminum tolerance in plant species. The major gene effect and allelic diversity at the Alt_{SB} locus can be exploited for improving Al tolerance of sensitive sorghum genotypes and other species.

36.3
Genetic and Genomics Resources of Sorghum

36.3.1
Germplasm Resources and Genetic Diversity

The plant genetic resources are defined as the "Genetic material of plants that is of value as a resource for the present and future generations of people" [121]. All

accessions of a particular crop species are expected to contain essentially the same genes. Differences in agricultural performance between accessions are thought to be due to allelic differences within the same gene set. Thus, genetic diversity in a crop is an important asset for improvement of its adaptive and agronomic traits. Genetic diversity is essential both for evolutionary history and for future evolutionary trajectory of a species. Most of the modern cultivars are having narrow genetic base making them vulnerable to potentially new biotic and abiotic stresses, the best example being the 1970 southern corn leaf blight (*Bipolarise maydays*) epidemic [122]. With a changing global climate scenario, exploitation and preservation of genetic diversity may become more evident for survival and sustainability of a crop.

Sorghum is a highly diverse species. There are three *S. bicolor* subspecies, cultivated types (ssp. *bicolor*), wild (ssp. *verticilliflorum*), and weedy types that are product of hybridization between domesticated and wild sorghums (ssp. *drummondii*). Furthermore, within ssp. *bicolor*, there are 5 races (i.e., bicolor, caudatum, durra, guinea, and kafir) and 10 intermediate races have been described on the basis of panicle and spikelet morphology [123]. Sorghum genetic resources are conserved at many centers around the world including India, China, the United States, Ethiopia, Sudan, and South Africa. At the global level, sorghum germplasm collections consist of approximately 168 500 accessions. International Crops Research Institute for Semi-Arid Tropics (ICRISAT), India, is a major repository for world sorghum germplasm with a total of 37 000 accessions from 91 countries [124]. To facilitate enhanced utilization of diverse germplasm in breeding program, a core collection of 2247 accessions was developed in 2001 [125]. As this core collection was found to be too large, a sorghum minicore with 242 accessions (10% of the core or 1% of the entire collection) was developed from the existing core collection [124]. A minicore collection thus may help in a precise evaluation and phenotyping for various traits.

Different molecular markers (RFLP, RAPD, AFLP, or SSR) have been used for molecular analysis of genetic diversity in sorghum germplasm [126–131]. These studies revealed that genetic diversity in sorghum is mostly influenced by racial and geographic origins [126, 127, 129, 131]. A worldwide core collection of 210 landraces representative of race, latitude of origin, response to day length, and production system was analyzed with 74 restriction fragment length polymorphism (RFLP) probes dispersed throughout the genome indicating that along with the geographical and racial genetic diversity, there were varying levels of diversity within specific morphological races. Among races, the highest diversity was exhibited by bicolor race and least by kafir [132].

36.3.2
Genetic Maps and QTL Mapping

Several studies identified QTL associated with various traits in sorghum including disease resistance [133], insect resistance [134], plant height and maturity [135], and drought tolerance (references given in Section 36.2.1). Two high-density linkage maps are also available [136, 137]. The linkage map created by Menz *et al*. [136] consists of 2926 loci on 10 linkage groups with a total genetic distance of 1713 cM, while map

developed by Bowers *et al*. [137] contained 2512 loci on 10 linkage groups, with a total genetic distance of 1059.2 cM. Later, these two maps have been aligned by identifying and mapping markers common to both populations [138]. On the basis of fluorescent *in situ* hybridization (FISH) of sorghum genomic BAC clones, a size-based nomenclature for sorghum chromosomes (SBI-01–SBI-10) and linkage groups (LG-01–LG-10) has been proposed [139]. A unified system of nomenclature for chromosome and linkage maps will benefit the validation and comparison of QTL across different backgrounds and environments. Recently, using the genome sequence more than 6500 simple sequence repeat (SSR) loci with publicly available primer sequences have been mapped *in silico* on sorghum genome [140]. This will facilitate the identification of markers representing the entire genome, which in turn will not only improve resolution in diversity analyses and linkage disequilibrium mapping but also help in fine mapping and marker-assisted breeding. Besides standard molecular markers such as RFLP and SSR, a new hybridization-based diversity array technology (DArT™) has also been developed for sorghum [141]. Recently, six-component mapping populations were used to integrate over 2000 unique loci, including 1190 unique DArT markers and 839 others, into a single consensus map with an average marker density of one marker/0.79 cM [142]. This consensus map, however, still has overall lower marker density compared to that one marker/0.59 cM and one marker/0.42 cM published by Menz *et al*. [136] and Bower *et al*. [137], respectively. DArT provides the advantage of being a cost-effective, high-throughput marker technology that is independent of sequence information and allows high multiplexing level for whole genome profiling.

36.3.3
Association Genetics

Association mapping, also called linkage disequilibrium (LD) mapping, refers to the analysis of statistical associations between genotypes, usually individual single-nucleotide polymorphisms (SNPs) or SNP haplotypes, determined in a collection of individuals, and the traits (phenotypes) of the same individuals [143]. First developed for human genetics, association genetics has now been used for dissecting complex traits in crop plants [144]. In plants, a collection of individuals refers to those that are derived from wild populations, germplasm collections, or subsets of breeding germplasm. The levels of genetic variation and linkage disequilibrium (LD) are critical factors both in association mapping and in identification of loci that have been targets of selection. Sorghum being largely a self-pollinating crop is expected to have higher levels of LD and homozygosity, which are suitable parameters for LD mapping [145]. Analysis of 27 diverse *S. bicolor* accessions for sequence variation at about 30 000 sites throughout the genome of *S. bicolor* indicates that the frequency of SNPs is about one-fourth of that observed in a comparable sample of maize accessions [146]. The extent of allelic associations, as assessed by pairwise measures of LD, is higher in *S. bicolor* than in maize, but lower than in rice and *Arabidopsis*. Hamblin *et al*. [147] demonstrated that in sorghum LD could extend up to 100 kb, but had largely decayed by 15 kb, meaning that targeted association mapping is possible in this species. To facilitate the association studies in sorghum, Casa *et al*. [148] have

characterized a panel of 377 accessions. These accessions were phenotyped for eight traits, and levels of population structure and familial relatedness were assessed with 47 SSR loci. The genotypic data for this panel along with appropriate statistical models for correcting for population structure and kinship are available for the entire sorghum community. Furthermore, efforts are being made to develop recombinant inbred populations for carrying out nested association mapping strategies in sorghum [148]. Recently, a few candidate gene-based association studies have been reported for various traits in sorghum such as plant height, brix, starch metabolism, and grain quality [149–152]. However, for complex quantitative traits such as drought stress, a genome-wide association mapping may be more useful. This will also require a genome-wide coverage of markers. Owing to their high density, SNPs play an important role in genome-scale linkage disequilibrium and association studies. About 1402 SNP alleles were reported by Hamblin *et al.* [146, 147, 151] through direct sequencing, while 2217 SNPs were detected in sorghum from analysis of loci from public EST databases [153].

36.3.4
Transcriptomics and Reverse Genetics

Besides sequence-based information, adaptive responses of sorghum have been monitored by genome-wide expression analysis under different stress conditions such as salinity, osmotic stress, or abscisic acid [154]. In addition, a sorghum Expressed Sequence Tags (ESTs) project has collected over 200 000 sequences from cDNA libraries derived from diverse tissues [155] and by December 2010, 209 828 ESTs were available at EST database of National Center for Biotechnological Information (NCBI). Various *in silico* genome-wide analyses of genes, promoters, or miRNAs are being performed that will help in identification and characterization of existing and new orthologues of these sequences [156, 157].

Additional resources for sorghum include mutant populations that are either being screened for target traits such epicuticular wax [158] or being developed as TILLING populations [159]. A TILLING population of 1600 lines has also been generated through EMS mutagenesis in sorghum genotype BTx 623 and its applicability has been evaluated on a subset of mutant lines [159]. Isolation of *Candystripe1* (*Cs1*), first active transposable element from sorghum, has potential for insertion mutagenesis and transposon tagging in sorghum [160]. The possibility of genetic transformation in sorghum [161–163] provides equal opportunities for both functional validation and crop improvement strategies. Moreover, the results of interspecific hybridization have been encouraging that will allow inclusion of allelic diversity in cultivated sorghum [164, 165].

36.3.5
Comparative Genomics

Besides using its own genetic and genomic resources, sorghum can be benefited by the high degree of genic colinearity and sequence conservation that prevails among

cereals [166, 167]. The syntenic relationship of sorghum with other cereals has become instrumental in the construction of genetic maps, verification of certain quantitative trait loci, identification of candidate genes underlying QTL, and genome evolution [109, 168–170]. Postgenome sequencing, enormous information is emerging from rice. The knowledge gained from rice can be used to accelerate progress in sorghum and sorghum in turn can benefit closely related large genomes such as maize and sugarcane. For example, analysis of miRNA in sorghum genome indicates that rice miRNA 169 g, which is upregulated during drought stress, has five sorghum homologues. Similarly, cytochrome P450 domain-containing genes, often involved in scavenging toxins such as those accumulated in response to stress, are more abundant in sorghum than in rice [4]. A detailed analysis of these duplicated genes may shed light on the adaptive nature of sorghum. On the other hand, sorghum genome has been found to be an excellent template for assembling the genic DNA of the autopolyploid sugarcane genome and Miscanthus × giganteus genome [171, 172]. Thus, with rice, sorghum and *Brachypodium distachyon* genome [173] sequences already available, and with impending maize genome sequence, there is an immense opportunity for comparative genetics and genomics to dissect abiotic stress tolerance mechanisms in cereals.

36.4 Prospects

Postgenome sequencing, there has been a phenomenal change in the prospects of sorghum research in general. The focus has been shifted to sorghum because of several inherent attributes that make it a highly promising system in this global climate change scenario. With the availability of whole-genome sequence, wide germplasm resource and diversity, high-density linkage maps, array of markers coupled with tolerance to drought and heat, and potential candidate as bioenergy crop, sorghum is poised for modeling a future crop. There are several traits that are best represented by sorghum and yet remain unexplored. For example, sorghum tends to arrest growth during periods of drought and grows rapidly when water is available, thus avoiding yield losses. The extensive root system of sorghum can penetrate 1.5–2.5 m into the soil and extend 1 m away from the stem. Roots harvest water and nutrients from soil and thus play an important role in adaptation to abiotic stresses. Several root QTL have been identified in rice and maize, yet no such efforts have been made in sorghum. Maybe the extensive root system of sorghum itself could pose difficulty in phenotyping. The availability of advanced phenotyping facilities and information generated from rice and maize root QTL studies can be exploited. Furthermore, sorghum apparently shows epicuticular wax values close to maximum that can be achieved by plants. Though genetic and chemical analyses of epicuticular wax mutants have been reported, molecular aspects are needed to be understood. Components such as membrane stability and water use efficiency require a thorough evaluation. Passioura [174] remarked, “Drought tolerance is a nebulous term that becomes more nebulous the more closely we look at it, much as a newspaper

photograph does when viewed through a magnifying glass." Thanks to the tremendous progress in understanding drought tolerance mechanisms during the past two decades, and the availability of high-throughput phenomics and genomics tools, today plant scientist hope that crop drought tolerance can be improved drop by drop, trait by trait, and gene by gene [175]. Hence, application of high-throughput "omics" approach to understand the abiotic stress-adaptive mechanisms of sorghum will help genetic improvement of abiotic stress tolerance in sorghum. The trait of seedling emergence and seed vigor under cold from Chinese landraces is associated with transfer of negative traits such as susceptibility to leaf diseases. Identification of QTL is being done; however, tightly linked markers need to be developed for precise introgression of this trait in elite cultivars. Though it has been claimed that sorghum is tolerant to heat, yet there is no systematic study to illustrate this trait in sorghum. Overall, the attributes of sorghum for abiotic stress tolerance are still unexplored. Though some physiological evidences are available and genetic studies have been initiated, yet detail understanding of molecular and physiological mechanism is necessary for improvement of sorghum and cereal family in general.

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References

1 Bengtsson, M., Shen, Y., and Oki, T. (2006) A SRES-based gridded global population dataset for 1990–2100. *Popul. Environ.*, **28** (2), 113–131.
2 Rosegrant, M.W. and Cline, S.A. (2003) Global food security: challenges and policies. *Science*, **302** (5652), 1917–1918.
3 Intergovernmental Panel on Climate Change (2007) Climate Change 2007: the Physical Science Basis. Contribution of Working Group I to the Fourth Annual Assessment Report of the IPCC.
4 Paterson, A.H., Bowers, J.E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, G., Hellsten, U., Mitros, T., Poliakov, A., Schmutz, J., Spannagl, M., Tang, H., Wang, X., Wicker, T., Bharti, A.K., Chapman, J., Feltus, F.A., Gowik, U., Grigoriev, I.V., Lyons, E., Maher, C.A., Martis, M., Narechania, A., Otillar, R.P., Penning, B.W., Salamov, A.A., Wang, Y., Zhang, L., Carpita, N.C., Freeling, M., Gingle, A.R., Hash, C.T., Keller, B., Klein, P., Kresovich, S., McCann, M.C., Ming, R., Peterson, D.G., Mehboob-ur-Rahman, Ware, D., Westhoff, P., Mayer, K.F., Messing, J., and Rokhsar, D.S. (2009) The *Sorghum bicolor* genome and the diversification of grasses. *Nature*, **457** (7229), 551–556.
5 Boyer, J.S. (1982) Plant productivity and environment. *Science*, **218** (4571), 443–448.
6 Araus, J.L., Slafer, G.A., Royo, C., and Serret, M.D. (2008) Breeding for yield potential and stress adaptation in cereals. *Crit. Rev. Plant Sci.*, **27** (6), 377–412.
7 Fischer, R.A. and Edmeades, G.O. (2010) Breeding and cereal yield progress. *Crop Sci.*, **50** (S1), S85–S98.

8 Reynolds, M.P. and Trethowan, R.M. (2007) Physiological interventions in breeding for adaptation to abiotic stress, in *Scale and Complexity in Plant Systems, Research: Gene–Plant-Crop Relations* (eds J.H.J. Spiertz, P.C. Struik, and H.H. van Laar), Springer, pp. 129–146.

9 Bennett, J. (2003) Opportunities for increasing water productivity of CGIAR crops through plant breeding and molecular Biology, in *Water Productivity in Agriculture: Limits and Opportunities for Improvement* (eds J.W. Kijne, R. Barker, and D. Molden), CAB International, pp. 103–126.

10 Rosenow, D.T., Quisenberry, J.E., Wendt, C.W., and Clark, L.E. (1983) Drought tolerant sorghum and cotton germplasm. *Agr. Water Manage.*, **7** (1–3), 207–222.

11 Sanchez, A.C., Subudhi, P.K., Rosenow, D.T., and Nguyen, H.T. (2002) Mapping QTLs associated with drought resistance in sorghum (*Sorghum bicolor* L. Moench). *Plant Mol. Biol.*, **48** (5–6), 713–726.

12 Borrell, A.K., Hammer, G.L., and Henzell, R.G. (2000) Does maintaining green leaf area in sorghum improve yield under drought? II. Dry matter production and yield. *Crop Sci.*, **40** (4), 1037–1048.

13 Tuinstra, M.R., Grote, E.M., Goldsbough, P.B., and Ejeta, G. (1996) Identification of quantitative trait loci associated with pre-flowering drought tolerance in sorghum. *Crop Sci.*, **36** (5), 1337–1344.

14 Kebede, H., Subudhi, P.K., Rosenow, D.T., and Nguyen, H.T. (2001) Quantitative trait loci influencing drought tolerance in grain sorghum (*Sorghum bicolor* L. Moench). *Theor. Appl. Genet.*, **103** (2–3), 266–276.

15 Blum, A. (2005) Drought resistance, water-use efficiency, and yield potential: are they compatible, dissonant, or mutually exclusive? *Aust. J. Agric. Res.*, **56** (11), 1159–1168.

16 Thomas, H. and Howarth, C.J. (2000) Five ways to stay green. *J. Exp. Bot.*, **51** (S1), 329–337.

17 Borrell, A.K. and Hammer, G.L. (2000) Nitrogen dynamics and the physiological basis of stay-green in sorghum. *Crop Sci.*, **40** (5), 1295–1307.

18 Borrell, A.K., Hammer, G.L., and Henzell, R.G. (2000) Does maintaining green leaf area in sorghum improve yield under drought? I. Leaf growth and senescence. *Crop Sci.*, **40** (4), 1026–1037.

19 Rosenow, D.T. (1984) Breeding for resistance to root and stalk rot in Texas, in *Sorghum Root and Stalk Diseases: A Critical Review* (ed. L.K. Mughogho), Proceedings of the consultative discussion of Research Needs and Strategies for Control of Sorghum Root and Stalk Diseases November 27–December 2 1983 Bellagio Italy, International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India, pp. 209–217.

20 Mahalakshmi, V. and Bidinger, F.R. (2002) Evaluation of stay-green sorghum germplasm lines at ICRISAT. *Crop Sci.*, **42** (3), 965–974.

21 Haussmann, B., Mahalakshmi, V., Reddy, B., Seetharama, N., Hash, C., and Geiger, H. (2002) QTL mapping of stay-green in two sorghum recombinant inbred populations. *Theor. Appl. Genet.*, **106** (1), 133–142.

22 Hash, C.T., Bhasker Raj, A.G., Lindup, S., Sharma, A., Beniwal, C.R., Folkertsma, R.T., Mahalakshmi, V., Zerbinid, E., and Blummeld, M. (2003) Opportunities for marker-assisted selection (MAS) to improve the feed quality of crop residues in pearl millet and sorghum. *Field Crops Res.*, **84** (1–2), 79–88.

23 VanOosterom, E.J., Jayachandran, R., and Bidinger, F.R. (1996) Diallel analysis of the stay-green trait and its components in sorghum. *Crop Sci.*, **36** (3), 549–555.

24 Walulu, R.S., Rosenow, D.T., Wester, D.B., and Nguyen, H.T. (1994) Inheritance of the stay green trait in sorghum. *Crop Sci.*, **34** (4), 970–972.

25 Tuinstra, M.R., Grote, E.M., Goldsbrough, P.B., and Ejeta, G. (1997) Genetic analysis of post-flowering drought tolerance and components of grain development of *Sorghum bicolor (L.) Moench*. *Mol. Breed.*, **3** (6), 439–448.

26 Crasta, O.R., Xu, W.W., Rosenow, D.T., Mullet, J., and Nguyen, H.T. (1999) Mapping of post-flowering drought

resistance traits in grain sorghum: association between QTLs influencing premature senescence and maturity. *Mol. Gen. Genet.*, **262** (3), 579–588.

27 Xu, W., Subudhi, P.K., Crasta, O.R., Rosenow, D.T., Mullet, J.E., and Nguyen, H.T. (2000) Molecular mapping of QTLs conferring staygreen in grain sorghum (*Sorghum bicolor* L. Moench). *Genome*, **43** (3), 461–469.

28 Subudhi, P.K., Rosenow, D.T., and Nguyen, H.T. (2000) Quantitative trait loci for the stay green trait in sorghum (*Sorghum bicolor* L. Moench): consistency across genetic backgrounds and environments. *Theor. Appl. Genet.*, **101** (5–6), 733–741.

29 Tao, Y.Z., Henzell, R.G., Jordan, D.R., Butler, D.G., Kelly, A.M., and McIntyre, C.L. (2000) Identification of genomic regions associated with stay-green in sorghum by testing RILs in multiple environments. *Theor. Appl. Genet.*, **100** (8), 1225–1232.

30 Harris, K., Subudhi, P.K., Borrell, A., Jordan, D., Rosenow, D., Nguyen, H., Klein, P., Klein, R., and Mullet, J. (2007) Sorghum stay-green QTL individually reduce post-flowering drought-induced leaf senescence. *J. Exp. Bot.*, **58** (2), 327–338.

31 Kassahun, B., Bidinger, F.R., Hash, C.T., and Kuruvinashetti, M.S. (2010) Stay-green expression in early generation sorghum [*Sorghum bicolor* (L.) Moench] QTL introgression lines. *Euphytica*, **172** (3), 351–362.

32 Srinivas, G., Satish, K., Murali Mohan, S., Nagaraja Reddy, R., Madhusudhana, R., Balakrishna, D., Venkatesh Bhat, B., Howarth, C.J., and Seetharama, N. (2008) Development of genic-microsatellite markers for sorghum staygreen QTL using a comparative genomic approach with rice. *Theor. Appl. Genet.*, **117** (2), 283–296.

33 Yoo, S.C., Cho, S.H., Zhang, H., Paik, H.C., Lee, C.H., Li, J., Yoo, J.H., Lee, B.W., Koh, H.J., Seo, H.S., and Paek, N.C. (2007) Quantitative trait loci associated with functional stay-green SNU-SG1 in rice. *Mol. Cells*, **24** (1), 83–94.

34 Kumar, U., Joshi, A.K., Kumari, M., Paliwal, R., Kumar, S., and Roder, M.S. (2010) Identification of QTLs for stay green trait in wheat (*Triticum aestivum* L.) in the "Chirya 3" X "Sonalika" population. *Euphytica*, **174** (3), 437–445.

35 Cha, K.W., Lee, Y.J., Koh, H.J., Lee, B.M., Nam, Y.W., and Paek, N.C. (2002) Isolation, characterization, and mapping of the stay green mutant in rice. *Theor. Appl. Genet.*, **104** (4), 526–532.

36 Guiamet, J.J., Schwartz, E., Pichersky, E., and Nooden, L.D. (1991) Characterization of cytoplasmic and nuclear mutations affecting chlorophyll and chlorophyll-binding proteins during senescence in soybean. *Plant Physiol.*, **96** (1), 227–231.

37 Akhtar, M.S., Goldschmidt, E., John, I., Rodoni, S., Matile, P., and Grierson, D. (1999) Altered patterns of senescence and ripening in gf, a stay-green mutant of tomato (*Lycopersicon esculentum* Mill). *J. Exp. Bot.*, **50** (336), 1115–1122.

38 Fang, Z., Bouwkamp, J.C., and Solomos, T. (1998) Chlorophyllase activities and chlorophyll degradation during leaf senescence in non yellowing mutant and wild type of *Phaseolus vulgaris* L. *J. Exp. Bot.*, **49** (320), 503–510.

39 Efrati, A., Eyal, Y., and Paran, I. (2005) Molecular mapping of the chlorophyll retainer (cl) mutation in pepper (*Capsicum* spp.) and screening for candidate genes using tomato ESTs homologous to structural genes of the chlorophyll catabolism pathway. *Genome*, **48** (2), 347–351.

40 Thomas, H. and Stoddart, J. (1975) Separation of chlorophyll degradation from other senescence processes in leaves of a mutant genotype of meadow fescue (*Festuca pratensis* L.). *Plant Physiol.*, **56** (3), 438–441.

41 Park, S.Y., Yu, J.W., Park, J.S., Li, J., Yoo, S.C., Lee, N.Y., Lee, S.K., Jeong, S.W., Seo, H.S., Koh, H.J., Jeon, J.S., Park, Y.I., and Paek, N.C. (2007) The senescence-induced staygreen protein regulates chlorophyll degradation. *Plant Cell*, **19** (5), 1649–1664.

42 Jiang, H.W., Li, M.R., Liang, N.B., Yan, H.B., Wei, Y.L., Xu, X., Liu, J.F., Xu, Z.,

Chen, F., and Wu, G.J. (2007) Molecular cloning and function analysis of the stay green gene in rice. *Plant J.*, **52** (2), 197–209.

43 Sato, Y., Morita, R., Nishimura, M., Yamaguchi, H., and Kusaba, M. (2007) Mendel's green cotyledon gene encodes a positive regulator of the chlorophyll-degrading pathway. *Proc. Natl. Acad. Sci. USA*, **104** (35), 14169–14174.

44 Barry, C.S., McQuinn, R.P., Chung, M.Y., Besuden, A., and Giovannoni, J.J. (2008) Amino acid substitutions in homologs of the stay-green protein are responsible for the green-flesh and chlorophyll retainer mutations of tomato and pepper. *Plant Physiol.*, **147** (1), 179–187.

45 Jenks, M.A. and Ashworth, E.N. (1999) Plant epicuticular waxes: function, production, and genetics, in *Horticultural Reviews*, vol. **23** (ed. J. Janick), John Wiley & Sons, Inc., New York, pp. 1–68.

46 Kosma, D.K. and Jenks, M.A. (2007) Eco-physiological and molecular-genetic determinants of plant cuticle function in drought and salt stress tolerance, in *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops* (eds M.A. Jenks, P.M. Hasegawa, and S.M. Jain), Springer, pp. 91–120.

47 Islam, M.A., Du, H., Ning, J., Ye, H., and Xiong, L. (2009) Characterization of Glossy1-homologous genes in rice involved in leaf wax accumulation and drought resistance. *Plant Mol. Biol.*, **70** (4), 443–456.

48 Jenks, M.A., Rich, P.J., Rhodes, D., Ashworth, E.N., Axtell, J.D., and Ding, C.K. (2000) Leaf sheath cuticular waxes on bloomless and sparse bloom mutants of *Sorghum bicolor*. *Phytochemistry*, **54** (6), 577–584.

49 Burow, G.B., Franks, C.D., and Xin, Z. (2008) Genetic and physiological analysis of an irradiated bloomless mutant (epicuticular wax mutant) of sorghum. *Crop Sci.*, **48** (1), 41–48.

50 O'Toole, J.C. and Cruz, R.T. (1983) Genotypic variation in epicuticular wax of rice. *Crop Sci.*, **23** (2), 392–394.

51 Beattie, G.A. and Marcell, L.M. (2002) Effect of alterations in cuticular wax biosynthesis on the physicochemical properties and topography of maize leaf surfaces. *Plant Cell Environ.*, **25** (1), 1–16.

52 Araus, J.L., Febrero, A., and Vendrell, P. (1991) Epidermal conductance in different parts of durum wheat grown under Mediterranean conditions: the role of epicuticular waxes and stomata. *Plant Cell Environ.*, **14** (6), 545–558.

53 Bianchi, A. and Bianchi, G. (1990) Surface lipid composition of C3 and C4 plants. *Biochem. Syst. Ecol.*, **18** (7–8), 533–537.

54 Ohlrogge, J.B. and Jaworski, J.G. (1997) Regulation of fatty acid synthesis. *Annu. Rev. Plant Physiol. Mol. Biol.*, **48**, 109–136.

55 Agrawal, V.P., Lessire, R., and Stumpf, P.K. (1984) Biosynthesis of very long chain fatty acids in microsomes of epidermal cells of *Allium porrum* L. *Arch. Biochem. Biophys.*, **230** (2), 580–589.

56 Samuels, L., Kunst, L., and Jetter, R. (2008) Sealing plant surfaces: cuticular wax formation by epidermal cells. *Annu. Rev. Plant Biol.*, **59**, 683–707.

57 Kunst, L. and Samuels, L. (2009) Plant cuticles shine: advances in wax biosynthesis and export. *Curr. Opin. Plant Biol.*, **12** (6), 721–727.

58 Sturaro, M., Hartings, H., Schmelzer, E., Velasco, R., Salamini, F., and Motto, M. (2005) Cloning and characterization of *GLOSSY1*, a maize gene involved in cuticle membrane and wax production. *Plant Physiol.*, **138** (1), 478–489.

59 Peterson, G.C., Suksayretrup, K., and Weibel, D.E. (1982) Inheritance of some bloomless and sparse-bloom mutants in sorghum. *Crop Sci.*, **22** (1), 63–67.

60 Jenks, M.A., Joly, R.J., Rich, P.J., Peters, P.J., Axtell, J.D., and Ashworth, E.N. (1994) Chemically induced cuticle mutation affecting epidermal conductance to water vapor and disease susceptibility in *Sorghum bicolor* (L.) Moench. *Plant Physiol.*, **105** (4), 1239–1245.

61 Burow, G.B., Franks, C.D., Acosta-Martinez, V., and Xin, Z. (2009) Molecular mapping and characterization of *BLMC*, a locus for profuse wax (bloom)

and enhanced cuticular features of Sorghum (*Sorghum bicolor* (L.) Moench.). *Theor. Appl. Genet.*, **118** (3), 423–431.

62 Rhodes, D. and Samaras, Y. (1994) Genetic control of osmoregulation in plants, in *Cellular and Molecular Physiology of Cell Volume Regulation* (ed. K. Strange), CRC Press, pp. 347–367.

63 Morgan, J.M. (1984) Osmoregulation and water stress in higher plants. *Annu. Rev. Plant Physiol.*, **35**, 299–319.

64 Basnayake, J., Cooper, M., Ludlow, M.M., Henzell, R.G., and Snell, P.J. (1995) Inheritance of osmotic adjustment to water stress in three grain sorghum crosses. *Theor. Appl. Genet.*, **90** (5), 675–682.

65 Chen, T.H.H. and Murata, N. (2008) Glycinebetaine: an effective protectant against abiotic stress in plants. *Trends Plant Sci.*, **13** (9), 499–505.

66 Wood, A.J., Saneoka, H., Rhodes, D., Joly, R.J., and Goldsbrough, P.B. (1996) Betaine aldehyde dehydrogenase in sorghum. *Plant Physiol.*, **110** (4), 1301–1308.

67 Rathinasabapathi, B., Gage, D.A., Mackill, D.J., and Hanson, A.D. (1993) Cultivated and wild rices do not accumulate glycinebetaine due to deficiencies in two biosynthetic steps. *Crop Sci.*, **33** (3), 534–538.

68 Yang, W.J., Nadolskaorczyk, A., Wood, K.V., Hahn, D.T., Rich, P.J., Wood, A.J., Saneoka, H., Premachandra, G.S., Bonham, C.C., Rhodes, J.C., Joly, R.J., Samaras, Y., Goldsbrough, P.B., and Rhodes, D. (1995) Near-isogenic lines of maize differing for glycinebetaine. *Plant Physiol.*, **107** (2), 621–630.

69 Mickelbart, M.V., Peel, G., Joly, R.J., Rhodes, D., Ejeta, G., and Goldsbrough, P.B. (2003) Development and characterization of near-isogenic lines of sorghum segregating for glycinebetaine accumulation. *Physiol. Plant.*, **118** (2), 253–261.

70 Peel, G.J., Mickelbart, M.V., and Rhodes, D. (2010) Choline metabolism in glycinebetaine accumulating and non-accumulating near-isogenic lines of *Zea mays* and *Sorghum bicolor*. *Phytochemistry*, **71** (4), 404–414.

71 Premachandra, G.S., Hahn, D.T., Rhodes, D., and Joly, R.J. (1995) Leaf water relations and solute accumulation in two grain sorghum lines exhibiting contrasting drought tolerance. *J. Exp. Bot.*, **46** (12), 1833–1841.

72 Doggett, H. (1988) *Sorghum*, 2nd edn, John Wiley & Sons, Inc., New York.

73 Yu, J. and Tuinstra, M.R. (2001) Genetic analysis of seedling growth under cold temperature stress in grain sorghum. *Crop Sci.*, **41** (5), 1438–1443.

74 Singh, S.P. (1985) Sources of cold tolerance in grain sorghum. *Can. J. Plant Sci.*, **65** (2), 251–257.

75 Cisse, N. and Ejeta, G. (2003) Genetic variation and relationships among seedling vigor traits in sorghum. *Crop Sci.*, **43** (3), 824–828.

76 Franks, C.D., Burow, G.B., and Burke, J.J. (2006) A comparison of U.S. and Chinese sorghum germplasm for early season cold tolerance. *Crop Sci.*, **46** (3), 1371–1376.

77 Knoll, J., Gunaratna, G., and Ejeta, G. (2008) QTL analysis of early-season cold tolerance in sorghum. *Theor. Appl. Genet.*, **116** (4), 577–587.

78 Knoll, J. and Ejeta, G. (2008) Marker-assisted selection for early-season cold tolerance in sorghum: QTL validation across populations and environments. *Theor. Appl. Genet.*, **116** (4), 541–553.

79 Chinnusamy, V. and Zhu, J.K. (2003) Plant salt tolerance, in *Plant Stress Responses: Topics in Current Genetics*, vol. **4** (eds H. Hirt and K. Shinozaki), Springer, pp. 241–270.

80 Maas, E.V. (1985) Crop tolerance to saline sprinkling water. *Plant Soil*, **89** (1–3), 273–284.

81 Igartua, E., Gracia, M.P., and Lasa, J.M. (1995) Field responses of grain sorghum to a salinity gradient. *Field Crops Res.*, **42** (1), 15–25.

82 Munns, R. and Tester, M. (2008) Mechanism of salinity. *Annu. Rev. Plant Biol.*, **59**, 651–681

83 Azhar, F.M. and McNeilly, T. (1988) The genetic basis of variation for salt tolerance in *Sorghum bicolor* (L.) Moench seedlings. *Plant Breed.*, **101** (2), 114–121.

84 Azhar, F.M. and McNeilly, T. (1987) Variability for salt tolerance in *Sorghum bicolor* (L.) Moench under hydroponic conditions. *J. Agron. Crop. Sci.*, **159** (4), 269–277.

85 Maiti, R.K., de la Rosa-Ibarra, M., and Sandowal, N.D. (1994) Genotypic variability in glossy sorghum lines for resistance to drought, salinity and temperature-stress at seedling stage. *J. Plant Physiol.*, **143** (2), 241–244.

86 Krishnamurthy, L., Serraj, R., Hash, C.T., Dakheel, A.J., and Reddy, B.V.S. (2007) Screening sorghum genotypes for salinity tolerant biomass production. *Euphytica*, **156** (15–24), 15–24.

87 Ma, J.F., Ryan, P.R., and Delhaize, E. (2001) Aluminum tolerance in plants and the complexing role of organic acids. *Trends Plant Sci.*, **6** (6), 273–278.

88 Ciamporova, M. (2002) Morphological and structural responses of plant roots to aluminum at organ, tissue and cellular levels. *Biol. Plant.*, **45** (2), 161–171.

89 Kochian, L.V., Hoekenga, O.A., and Pineros, M.A. (2004) How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. *Annu. Rev. Plant Biol.*, **55**, 459–493.

90 Van Uexküll, H.R. and Mutert, E. (1995) Global extent, development and economic impact of acid soils. *Plant Soil*, **171** (1), 1–15.

91 Ma, J.F., Nagao, S., Sato, K., Ito, H., Furukawa, J., and Takeda, K. (2004) Molecular mapping of a gene responsible for Al-activated secretion of citrate in barley. *J. Exp. Bot.*, **55** (401), 1335–1341.

92 Nguyen, V.T., Nguyen, B.D., Sarkarung, S., Martinez, C., Paterson, A.H., and Nguyen, H.T. (2002) Mapping of genes controlling aluminum tolerance in rice: comparison of different genetic backgrounds. *Mol. Genet. Genomics*, **267** (6), 772–780.

93 Tang, Y., Garvin, D.F., Kochian, L.V., Sorrells, M.E., and Carver, B.F. (2002) Physiological genetics of aluminum tolerance in the wheat cultivar Atlas 66. *Crop Sci.*, **42** (5), 1541–1546.

94 Aniol, A. and Gustafson, J.P. (1984) Chromosome location of genes controlling aluminum tolerance in wheat, rye and triticale. *Can. J. Genet. Cytol.*, **26** (6), 701–705.

95 Aniol, A. (1990) Genetics of tolerance to aluminum in wheat (*Triticum aestivum* L. Thell). *Plant Soil*, **123** (2), 223–227.

96 Papernik, L.A., Bethea, A.S., Singleton, T.E., Magalhaes, J.V., Garvin, D.F., and Kochian, L.V. (2001) Physiological basis of reduced Al tolerance in ditelosomic lines of Chinese Spring wheat. *Planta*, **212** (5–6), 829–834.

97 Magalhaes, J.V. (2010) How a microbial drug transporter became essential for crop cultivation on acid soils: aluminium tolerance conferred by the multidrug and toxic compound extrusion (MATE) family. *Ann. Bot.*, **106** (1), 199–203.

98 Basu, U., Basu, A., and Taylor, G.J. (1994) Differential exudation of polypeptides by roots of aluminum-resistant and aluminum-sensitive cultivars of *Triticum aestivum* L. in response to aluminum stress. *Plant Physiol.*, **106** (1), 151–158.

99 Jones, D.L. (1998) Organic acids in the rhizosphere: a critical review. *Plant Soil*, **205** (1), 25–44.

100 Jones, D.L. and Ryan, P.R. (2004) Nutrition. Aluminum toxicity. *Encyclopedia of Applied Plant Science*, pp. 656–664.

101 Delhaize, E., Ryan, P.R., and Randall, P.J. (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.) II. Aluminum stimulated excretion of malic acid from root apices. *Plant Physiol.*, **103** (3), 695–702.

102 Pellet, D.M., Grunes, D.L., and Kochian, L.V. (1995) Organic acid exudation as an aluminum-tolerance mechanism in maize (*Zea mays* L.). *Planta*, **196** (4), 788–795.

103 Yang, Z.M., Sivaguru, M., Horst, W.J., and Matsumoto, H. (2001) Aluminum tolerance is achieved by exudation of citric acid from roots of soybean (*Glycine max*). *Physiol. Plant.*, **110** (1), 72–74.

104 Zheng, S.J., Ma, J.F., and Matsumoto, H. (1998) High aluminum resistance in buckwheat. I. Al-induced specific secretion of oxalic acid from root tips. *Plant Physiol.*, **117** (3), 745–751.

105 Ma, Z. and Miyasaka, S.C. (1998) Oxalate exudation by taro in response to Al. *Plant Physiol.*, **118**, 861–865.

106 Li, X.F., Ma, J.F., and Matsumoto, H. (2000) Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiol.*, **123** (4), 1537–1543.

107 Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S.J., Ryan, P.R., Delhaize, E., and Matsumoto, H. (2004) A wheat gene encoding an aluminum-activated malate transporter. *Plant J.*, **37** (5), 645–653.

108 Raman, H., Zhang, K., Cakir, M., Appels, R., Garvin, D.F., Maron, L.G., Kochian, L.V., Moroni, J.S., Raman, R., Imtiaz, M., Drake-Brockman, F., Waters, I., Martin, P., Sasaki, T., Yamamoto, Y., Matsumoto, H., Hebb, D.M., Delhaize, E., and Ryan, P.R. (2005) Molecular characterization and mapping of ALMT1, the aluminium tolerance gene of bread wheat (*Triticum aestivum* L.). *Genome*, **48** (5), 781–791.

109 Magalhaes, J.V., Garvin, D.F., Wang, Y., Sorrells, M.E., Klein, P.E., Shaffert, R.E., Li, L., and Kochian, L.V. (2004) Comparative mapping of a major aluminum tolerance gene in sorghum and other species in the Poaceae. *Genetics*, **167** (4), 1905–1914.

110 Ma, J.F., Shen, R., Zhao, Z., Wissuwa, M., Takeuchi, Y., Ebitani, T., and Yano, M. (2002) Response of rice to Al stress and identification of quantitative trait loci for Al tolerance. *Plant Cell Physiol.*, **43** (6), 652–659.

111 Magalhaes, J.V., Liu, J., Guiamaraes, C.T., Lana, U.G.P., Alves, V.M.C., Wang, Y.H., Shaffert, R.E., Hoekenga, O.A., Pineros, M.A., Shaff, J.E., Klein, P.E., Carneiro, N.P., Coelho, C.M., Trick, H.N., and Kochian, L.V. (2007) A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. *Nat. Genet.*, **39** (9), 1156–1161.

112 Furukawa, J., Yamaji, N., Wang, H., Mitani, N., Murata, Y., Sato, K., Katsuhara, M., Takeda, K., and Ma, J.F. (2007) An aluminum-activated citrate transporter in barley. *Plant Cell Physiol.*, **48** (8), 1081–1091.

113 Yokosho, K., Yamaji, N., Ueno, D., Mitani, N., and Ma, J.F. (2009) OsFRDL1 is a citrate transporter required for efficient translocation of iron in rice. *Plant Physiol.*, **149** (1), 297–305.

114 Liu, J., Magalhaes, J.V., Shaff, J., and Kochian, L.V. (2009) Aluminum-activated citrate and malate transporters from the MATE and ALMT families function independently to confer *Arabidopsis* aluminum tolerance. *Plant J.*, **57** (3), 389–399.

115 Maron, L.G., Piñeros, M.A., Guimarães, C.T., Magalhaes, J.V., Pleiman, J.K., Mao, C., Shaff, J., Silvia, N.J., Belicuas, S.N.J., and Kochian, L.V. (2009) Two functionally distinct members of the MATE (multidrug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *Plant J.*, **61** (5), 728–740.

116 Ryan, P.R., Raman, H., Gupta, S., Horst, W.J., and Delhaize, E. (2009) A second mechanism for aluminum resistance in wheat relies on the constitutive efflux of citrate from roots. *Plant Physiol.*, **149** (1), 340–351.

117 Ninamango-Cárdenas, F.E., Guimarães, C.T., Martins, P.R., Parentoni, S.N., Carneiro, N.P., Lopes, M.A., Moro, J.R., and Paiva, E. (2003) Mapping QTLs for aluminum tolerance in maize. *Euphytica*, **130** (2), 223–232.

118 Wight, C.P., Kibite, S., Tinker, N.A., and Molnar, S.J. (2006) Identification of molecular markers for aluminium tolerance in diploid oat through comparative mapping and QTL analysis. *Theor. Appl. Genet.*, **112** (2), 222–231.

119 Matos, M., Camacho, M.V., Perez-Flores, V., Pernaute, B., Pinto-Carnide, O., and Benito, C. (2005) A new aluminum tolerance gene located on rye chromosome arm 7RS. *Theor. Appl. Genet.*, **111** (2), 360–369.

120 Caniato, F.F., Guimarães, C.T., Schaffert, R.E., Alves, V.M., Kochian, L.V., Borém, A., Klein, P.E., and Magalhaes, J.V. (2007) Genetic diversity for aluminum tolerance

in sorghum. *Theor. Appl. Genet.*, **114** (5), 863–876.

121 International Plant Genetic Resources Institute (1993) Diversity For Development: IPGRI's First Strategy Report, IPGRI, Rome, Italy.

122 Tatum, L.A. (1971) The southern corn leaf blight epidemic. *Science*, **171** (3976), 1113–1116.

123 Harlan, J.R. and De Wet, J.M.J. (1972) A simplified classification of cultivated sorghum. *Crop Sci.*, **12** (2), 127–176.

124 Upadhyaya, H.D., Pundir, R.P.S., Dwivedi, S.L., Gowda, C.L.L., Reddy, V.G., and Singh, S. (2009) Developing a mini core collection of sorghum for diversified utilization of germplasm. *Crop Sci.*, **49** (5), 1769–1780.

125 Grenier, C., Bramel-Cox, P.J., and Hamon, P. (2001) Core collection of sorghum: I. Stratification based on eco-geographical data. *Crop Sci.*, **41** (1), 234–240.

126 Deu, M., Gonzalez-de-Leon, D., Glaszmann, J.C., Degremont, I., Chantereau, J., Lanaud, C., and Hamon, P. (1994) RFLP diversity in cultivated sorghum in relation to racial differentiation. *Theor. Appl. Genet.*, **88** (6–7), 838–844.

127 de Oliveira, A.C., Richter, T., and Bennetzen, J.L. (1996) Regional and racial specificities in sorghum germplasm assessed with DNA markers. *Genome*, **39** (3), 579–587.

128 Dahlberg, J.A., Zhang, X., Hart, G.E., and Mullet, J.E. (2002) Comparative assessment of variation among sorghum germplasm accessions using seed morphology and RAPD measurements. *Crop Sci.*, **42** (1), 291–296.

129 Agrama, H.A. and Tuinstra, M.R. (2003) Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. *Afr. J. Biotechnol.*, **10** (2), 334–340.

130 Menz, M.A., Klein, R.R., Unruh, N.C., Rooney, W.L., Klein, P.E., and Mullet, J.E. (2004) Genetic diversity of public inbreds of sorghum determined by mapped AFLP and SSR markers. *Crop Sci.*, **44** (4), 1236–1244.

131 Deu, M., Rattunde, F., and Chantereau, J. (2006) A global view of genetic diversity in cultivated sorghums using a core collection. *Genome*, **49** (2), 168–180.

132 Casa, A.M., Mitchell, S.E., Hamblin, M.T., Sun, H., Bowers, J.E., Paterson, A.H., Aquadro, C.F., and Kresovich, S. (2005) Diversity and selection in sorghum: simultaneous analyses using simple sequence repeats. *Theor. Appl. Genet.*, **111** (1), 23–30.

133 Murali Mohan, S., Madhusudhana, R., Mathur, K., Chakravarthi, D.V.N., Rathore, S., Nagaraja Reddy, R., Satish, K., Srinivas, G., Sarada Mani, N., and Seetharama, N. (2010) Identification of quantitative trait loci associated with resistance to foliar diseases in sorghum [*Sorghum bicolor* (L.) Moench]. *Euphytica*, **176** (2), 199–211.

134 Satish, K., Srinivas, G., Madhusudhana, R., Padmaja, P.G., Nagaraja Reddy, R., Murali Mohan, S., and Seetharama, N. (2009) Identification of quantitative trait loci for resistance to shoot fly in sorghum (*Sorghum bicolor* (L.) Moench). *Theor. Appl. Genet.*, **119** (8), 1425–1439.

135 Pereira, M.G. and Lee, M. (1995) Identification of genomic regions affecting plant height in sorghum and maize. *Theor. Appl. Genet.*, **90** (3–4), 380–388.

136 Menz, M.A., Klein, R.R., Mullet, J.E., Obert, J.A., Unruh, N.C., and Klein, P.E. (2002) A high-density genetic map of *Sorghum bicolor* (L.) Moench based on 2926 AFLP, RFLP and SSR markers. *Plant Mol. Biol.*, **48** (5–6), 483–499.

137 Bowers, J.E., Abbey, C., Anderson, S., Chang, C., Draye, X., Hoppe, A.H., Jessup, R., Lemke, C., Lennington, J., Li, Z.K., Lin, Y.R., Liu, S.C., Luo, L.J., Marler, B.S., Ming, R.G., Mitchell, S.E., Qiang, D., Reischmann, K., Schulze, S.R., Skinner, D.N., Wang, Y.W., Kresovich, S., Schertz, K.F., and Paterson, A.H. (2003) A high-density genetic recombination map of sequence-tagged sites for Sorghum, as a framework for comparative structural and evolutionary genomics of tropical grains and grasses. *Genetics*, **165** (1), 367–386.

138 Feltus, F.A., Hart, G.E., Schertz, K.F., Casa, A.M., Kresovich, S., Abraham, S., Klein, P.E., Brown, P.J., and Paterson, A.H. (2006) Alignment of genetic maps and QTLs between inter- and intra-specific sorghum populations. *Theor. Appl. Genet.*, **112** (7), 1295–1305.

139 Kim, J.S., Klein, P.E., Klein, R.R., Price, H.J., Mullet, J.E., and Stelly, D.M. (2005) Chromosome identification and nomenclature of *Sorghum bicolor*. *Genetics*, **169** (2), 1169–1173.

140 Ramu, P., Deshpande, S.P., Senthilvel, S., Jayashree, B., Billot, C., Deu, M., Ananda Reddy, L., and Hash, C.T. (2010) *In silico* mapping of important genes and markers available in the public domain for efficient sorghum breeding. *Mol. Breed.*, **26** (3), 409–418.

141 Mace, E.S., Xia, L., Jordan, D.R., Halloran, K., Parh, D.K., Huttner, E., Wenzl, P., and Kilian, A. (2008) DArT markers: diversity analyses and mapping in *Sorghum bicolor*. *BMC Genomics*, **9**, 26.

142 Mace, E.S., Rami, J.F., Bouchet, S., Klein, P.P., Klein, R.E., Kilian, A., Wenzl, P., Xia, L., Sakrewski, K., and Jordan, D.R. (2009) A consensus genetic map of sorghum that integrates multiple component maps and high-throughput Diversity Array Technology (DArT) markers. *BMC Plant Biol.*, **9**, 13.

143 Rafalski, J.A. (2010) Association genetics in crop improvement. *Curr. Opin. Plant Biol.*, **13** (2), 174–180.

144 Flint-Garcia, S.A., Thornsberry, J.M., and Buckler, E.S.I. (2003) Structure of linkage disequilibrium in plants. *Annu. Rev. Plant Biol.*, **54**, 357–374.

145 Nordborg, M., Borevitz, J.O., Bergelson, J., Berry, C.C., Chory, J., Hagenblad, J., Kreitman, M., Maloof, J.N., Noyes, T., Oefner, P.J., Stahl, E.A., and Weigel, D. (2002) The extent of linkage disequilibrium in *Arabidopsis thaliana*. *Nat. Genet.*, **30** (2), 190–193.

146 Hamblin, M.T., Mitchell, S.E., White, G.M., Gallego, J., Kukatla, R., Wing, R.A., Paterson, A.H., and Kresovich, S. (2004) Comparative population genetics of the panicoid grasses: sequence polymorphism, linkage disequilibrium and selection in a diverse sample of *Sorghum bicolor*. *Genetics*, **167** (1), 471–483

147 Hamblin, M.T., Salas Fernandez, M.G., Casa, A.M., Mitchell, S.E., Paterson, A.H., and Kresovich, S. (2005) Equilibrium processes cannot explain high levels of short- and medium-range linkage disequilibrium in the domesticated grass *Sorghum bicolor*. *Genetics*, **171** (3), 1247–1256.

148 Casa, A.M., Pressoir, G., Brown, P.J., Mitchell, S.E., Rooney, W.L., Tuinstra, M.R., Franks, C.D., and Kresovich, S. (2008) Community resources and strategies for association mapping in sorghum. *Crop Sci.*, **48** (1), 30–40.

149 Murray, S.C., Rooney, W.L., Hamblin, M.T., Mitchell, S.E., and Kresovich, S. (2009) Sweet sorghum genetic diversity and association mapping for brix and height. *Plant Genome*, **2** (1), 48–62.

150 Brown, B.J., Rooney, W.L., Franks, C., and Kresovich, S. (2008) Efficient mapping of plant height quantitative trait loci in a sorghum association population with introgressed dwarfing genes. *Genetics*, **180** (1), 629–637.

151 Hamblin, M.T., Salas Fernandez, M.G., Tuinstra, M.R., Rooney, W.L., and Kresovich, S. (2007) Sequence variation at candidate loci in the starch metabolism pathway in sorghum: prospects for linkage disequilibrium mapping. *Crop Sci.*, **47** (S2), S125–S134

152 De Alencar Figueiredo, L.F., Sine, B., Chantereau, J., Mestres, C., Fliedel, G., Rami, J.F., Glaszmann, J.C., Deu, M., and Courtois, B. (2010) Variability of grain quality in sorghum: association with polymorphism in Sh2, Bt2, SssI, Ae1, Wx and O2. *Theor. Appl. Genet.*, **121** (6), 1171–1185.

153 Feltus, F.A., Wan, J., Schulze, S.R., Estill, J.C., Jiang, N., and Paterson, A.H. (2004) An SNP resource for rice genetics and breeding based on subspecies indica and japonica genome alignments. *Genome Res.*, **14** (9), 1812–1819.

154 Buchanan, C.D., Lim, S., Salzman, R.A., Kagiampakis, I., Morishige, D.T., Weers, B.D., Klein, R.R., Pratt, L.H., Cordonnier-Pratt, M.M., Klein, P.E., and Mullet, J.E.

(2005) Sorghum bicolor's transcriptome response to dehydration, ABA, and high salt. *Plant Mol. Biol.*, **58** (5), 699–720.

155 Pratt, L.H., Liang, C., Shah, M., Sun, F., Wang, H., Reid, S.P., Gingle, A.R., Paterson, A.H., Wing, R., Dean, R., Klein, R., Nguyen, H.T., Ma, H.M., Zhao, X., Morishige, D.T., Mullet, J.E., and Cordonnier-Pratt, M.M. (2005) Sorghum expressed sequence tags identify signature genes for drought, pathogenesis, and skotomorphogenesis from a milestone set of 16,801 unique transcripts. *Plant Physiol.*, **139** (2), 869–884.

156 Du, J.F., Wu, Y.J., Fang, X.F., Cao, J.X., Zhao, L., and Tao, S.H. (2010) Prediction of sorghum miRNAs and their targets with computational methods. *Chin. Sci. Bull.*, **55** (13), 1263–1270.

157 Wang, S.K., Bai, Y.H., Shen, C.J., Wu, Y.R., Zhang, S.N., Jiang, D.A., Guilfoyle, T.J., Chen, M., and Qi, Y.H. (2010) Auxin-related gene families in abiotic stress response in *Sorghum bicolor*. *Funct. Integr. Genomics*, **10** (4), 533–546.

158 Peters, P.J., Jenks, M.A., Rich, P.J., Axtell, J.D., and Ejeta, J. (2009) Mutagenesis, selection, and allelic analysis of epicuticular wax mutants in sorghum. *Crop Sci.*, **49** (4), 1250–1258.

159 Xin, Z., Wang, M.L., Barkley, N.A., Burow, G., Franks, C., Pederson, G., and Burke, J. (2008) Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biol.*, **8**, 103.

160 Chopra, S., Brendel, V., Zhang, J., Axtell, J.D., and Peterson, T. (1999) Molecular characterization of a mutable pigmentation phenotype and isolation of the first active transposable element from *Sorghum bicolor*. *Proc. Natl. Acad. Sci. USA*, **96** (26), 15330–15335.

161 Visarada, K.B.R.S., Saikishore, N., Kuriakose, S.V., Shobha Rani, V., Royer, M., Rao, S.V., and Seetharama, N. (2008) A simple model for selection and rapid advancement of transgenic progeny in sorghum. *Plant Biotechnol. Rep.*, **2** (1), 47–58.

162 Raghuwanshi, A. and Birch, R.G. (2010) Genetic transformation of sweet sorghum. *Plant Cell Rep.*, **29** (9), 997–1005.

163 Saikishore, N., Visarada, K.B.R.S., Rao, S.V., and Seetharama, N. (2011) Progress and prospectus for *Agrobacterium*-mediated genetic transformation in sorghum in comparison with other cereals. *Transgenic Plant J.*, **5** (1), 27–34.

164 Price, H.J., Hodnett, G.L., Burson, B.L., Dillon, S.L., Stelly, D.M., and Rooney, W.L. (2006) Genotype dependent interspecific hybridization of sorghum bicolor. *Crop Sci.*, **46** (6), 2617–2622.

165 Hodnett, G.L., Hale, A.L., Packer, D.J., Stelly, D.M., Silva, J.D., and Rooney, W.L. (2010) Elimination of a reproductive barrier facilitates intergeneric hybridization of *Sorghum bicolor* and *Saccharum*. *Crop Sci.*, **50** (4), 1188–1195.

166 Gale, M.D. and Devos, K.M. (1998) Comparative genetics in the grasses. *Proc. Natl. Acad. Sci. USA*, **95** (5), 1971–1974.

167 Bennetzen, J.L. and Ma, J. (2003) The genetic colinearity of rice and other cereals on the basis of genomic sequence analysis. *Curr. Opin. Plant Biol.*, **6** (2), 128–133.

168 Pereira, M.G., Lee, M., Bramelcox, P., Woodman, W., Doebley, J., and Whitkus, R. (1994) Construction of an RFLP map in sorghum and comparative mapping in maize. *Genome*, **37** (2), 236–243.

169 Lin, Y.R., Schertz, K.F., and Paterson, A.H. (1995) Comparative analysis of QTLs affecting plant height and maturity across the Poaceae, in reference to an interspecific sorghum population. *Genetics*, **141** (1), 391–411.

170 Paterson, A.H., Lin, Y.R., Li, Z.K., Schertz, K.F., Doebley, J.F., Pinson, S.R.M., Liu, S.C., Stansel, J.W., and Irvine, J.E. (1995) Convergent domestication of cereal crops by independent mutations at corresponding genetic loci. *Science*, **269** (5231), 1714–1718.

171 Wang, J., Roe, B., Macmil, S., Yu, Q., Murray, J.E., Tang, H., Chen, C., Najar, F., Wiley, G., Bowers, J., Sluys, M.V., Rokhsar, D.S., Hudson, M.E., Moose, S.P., Paterson, A.H., and Ming, R. (2010) Microcollinearity between autopolyploid sugarcane and diploid sorghum genomes. *BMC Genomics*, **11**, 261.

172 Swaminathan, K., Alabady, M.S., Varala, K., De Paoli, E., Ho, I., Rokhsar, D.S., Arumuganathan, A.K., Ming, R., Green, P.J., Meyers, B.C., Moose, S.P., and Hudson, M.E. (2010) Genomic and small RNA sequencing of Miscanthus×giganteus shows the utility of sorghum as a reference genome sequence for Andropogoneae grasses. *Genome Biol.*, **11** (2), R12.

173 Vogel, J.P., Garvin, D.F., Mockler, T.C., Schmutz, J., Rokhsar, D., and Bevan, M.W. (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature*, **463** (7282), 763–768.

174 Passioura, J.B. (1996) Drought and drought tolerance. *Plant Growth Regul.*, **20**, 79–83.

175 Pennisi, E. (2008) The blue revolution, drop by drop, gene by gene. *Science*, **320** (5873), 171–173.

Section IIIB Fruit and Vegetable Crops

Improving Crop Resistance to Abiotic Stress, First Edition.
Edited by Narendra Tuteja, Sarvajeet Singh Gill, Antonio F. Tiburcio, and Renu Tuteja
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37
Vegetable Crops (Chili Pepper and Onion): Approaches to Improve Crop Productivity and Abiotic Stress Tolerance

Nandkumar Kunchge, Kiran Kumar, and Prashant Firke

The changing environments pose serious challenges to global agriculture and place unprecedented pressures on the sustainability of agriculture production. Climatic changes have been influencing the severity of environmental stress imposed on vegetable crops. Vegetable production is threatened by various abiotic stresses such as increased soil salinity, drought, temperature fluctuations, and so on. Success in breeding for varieties better adapted to abiotic stresses depends upon the concerted efforts by various research domains including plant and cell physiology, molecular biology, genetics, and breeding. The use of modern molecular biology tools for elucidating the control mechanisms of abiotic stress tolerance and for engineering stress-tolerant crops is based on the expression of specific stress-related genes. In this chapter, we have reviewed the studies reported on various factors responsible for abiotic stress in plants especially in vegetables with reference to chili pepper and onion. Physiological and molecular changes at genetic level during the stress response of plants are discussed briefly. This chapter emphasizes on the recent efforts to improve abiotic stress tolerance in crop plants by employing some of the stress-related genes and transcription factors in chili pepper and onion plants. The conventional breeding methods can be complemented by an array of biotechnological tools to augment vegetable production by saving time and resources. Molecular and genomics analyses have facilitated gene discovery and enabled genetic engineering using several functional or regulatory genes to activate specific or broad pathways related to abiotic stress tolerance in plants. There is a clear and urgent need to introduce stress tolerance genes into crop plants, in addition to establishing gene stacking or gene pyramiding.

37.1
Introduction

The human society has developed for thousands of years under one climatic state, and now a new set of climatic conditions are taking shape. These conditions are

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consistently warmer, and some areas are likely to see more extreme events like severe drought, torrential rain, and violent storms. While year-to-year changes in temperature often reflect natural climatic variations such as El Niño/La Niña events, changes in average temperature from decade to decade reveal long-term trends such as global warming.

Global warming and climate change have been widely accepted as facts. The changing environments pose serious challenges to global agriculture and place unprecedented pressures on the sustainability of horticulture industry. Adapting horticulture to future conditions is essential to meet the need of growing population and increasing demand for fruits, vegetables, and other horticultural products. Therefore, the development of horticultural crops that can cope with heat, cold, drought, and other climate extremes brought by a warming planet may well be the single most important step we can take to adapt to the changes we face today and in the future. In addition, it is estimated that more than 20% of all cultivated lands around the world contain levels of salts high enough to cause stress on crop plants, a situation worsened by global warming.

The tropical vegetable production environment is a mixture of conditions that varies with season and region. Climatic changes will influence the severity of environmental stress imposed on vegetable crops. Moreover, increasing temperatures, reduced irrigation water availability, flooding, and salinity will be major limiting factors in sustaining and increasing vegetable productivity. Extreme climatic conditions will also negatively impact soil fertility and increase soil erosion. Thus, additional fertilizer application or improved nutrient-use efficiency of crops will be needed to maintain productivity or harness the potential for enhanced crop growth due to increased atmospheric CO_2. The response of plants to environmental stresses depends on the plant developmental stage and the length and severity of the stress [1]. Plants may respond similarly to avoid one or more stresses through morphological or biochemical mechanisms [2]. Environmental interactions may make the stress response of plants more complex or influence the degree of impact of climate change.

37.2 Effect of Salinity

As the earth becomes less fertile after increased salinization of soil and water, in addition to drought, the importance of finding a way to improve crop yields will be vital to the survival of humans [3]. Drought and salinity are becoming increasingly prevalent such that severe salinization encompassing more than 50% of all cultivable land is projected by 2050 [4]. Vegetable production is threatened by increasing soil salinity particularly in irrigated croplands that provide 40% of the world's food. Excessive soil salinity reduces productivity of many agricultural crops, including most vegetables that are particularly sensitive throughout the ontogeny of the plant. According to the United States Department of Agriculture (USDA), onions are

sensitive to saline soils, while cucumbers, eggplants, peppers, and tomatoes, among the main crops of AVRDC – The World Vegetable Center, are moderately sensitive. In hot and dry environments, high evapotranspiration results in substantial water loss, thus leaving salt around the plant roots that interferes with the plant's ability to uptake water. Physiologically, salinity imposes an initial water deficit that results from the relatively high solute concentrations in the soil, causes ion-specific stresses resulting from altered K^+/Na^+ ratios, and leads to a buildup in Na^+ and Cl^- concentrations that are detrimental to plants [5].

Plant sensitivity to salt stress is reflected in loss of turgor, growth reduction, wilting, leaf curling and epinasty, leaf abscission, decreased photosynthesis, respiratory changes, loss of cellular integrity, tissue necrosis, and potentially death of the plant [6, 7]. Salinity also affects agriculture in coastal regions that are impacted by low-quality and high-saline irrigation water due to contamination of the groundwater and intrusion of saline water as a result of natural or manmade events. Salinity fluctuates with season, generally high in the dry season and low during rainy season when freshwater flushing is prevalent. Furthermore, coastal areas are threatened by specific, saline natural disasters that can make agricultural lands unproductive, such as tsunamis, which may inundate low-lying areas with seawater. Although the seawater rapidly recedes, the groundwater contamination and subsequent osmotic stress cause crop losses and affect soil fertility. In the inland areas, traditional water wells are commonly used for irrigation water in many countries. The bedrock deposit contains salts and the water from these wells is becoming more saline, thus affecting irrigated vegetable production in these areas.

37.3
Effect of High Temperature on Abiotic Stress

Temperature limits the range and production of many crops. In the tropics, high-temperature conditions are often prevalent during the growing season and, with a changing climate, crops in this area will be subjected to increased temperature stress. Analysis of climate trends in tomato-growing locations suggests that temperatures are rising and the severity and frequency of above-optimal temperature episodes will increase in the coming decades [8]. Vegetative and reproductive processes in tomatoes are strongly modified by temperature alone or in conjunction with other environmental factors [9]. High-temperature stress disrupts the biochemical reactions fundamental for normal cell function in plants. It primarily affects the photosynthetic functions of higher plants [10]. High temperatures can cause significant losses in tomato productivity due to reduced fruit set, and smaller and lower quality fruits [11]. Preanthesis temperature stress is associated with developmental changes in the anthers, particularly irregularities in the epidermis and endothesium, lack of opening of the stromium, and poor pollen formation [12]. Hazra *et al.* [13] summarized the symptoms causing fruit set failure at high temperatures in tomato; these include bud drop, abnormal flower development, poor pollen production,

dehiscence, and viability, ovule abortion and poor viability, reduced carbohydrate availability, and other reproductive abnormalities. In addition, significant inhibition of photosynthesis occurs at temperatures above optimum, resulting in considerable loss of potential productivity.

37.4
Abiotic Stress in Drought Conditions

Unpredictable drought is the single most important factor affecting world food security and the catalyst of the great famines of the past [14]. The world's water supply is fixed, thus increasing population pressure and competition for water resources will make the effect of successive droughts more severe [15]. Inefficient water usage all over the world and inefficient distribution systems in developing countries further decreases water availability. Water availability is expected to be highly sensitive to climate change and severe water stress conditions will affect crop productivity, particularly that of vegetables. In combination with elevated temperatures, decreased precipitation could cause reduction of irrigation water availability and increase in evapotranspiration, leading to severe crop water stress conditions [16].

Vegetables, being succulent products by definition, generally consist of greater than 90% water [17]. Thus, water greatly influences the yield and quality of vegetables; drought conditions drastically reduce vegetable productivity. Drought stress causes an increase in solute concentration in the environment (soil), leading to an osmotic flow of water out of plant cells. This leads to an increase in the solute concentration in plant cells, thereby lowering the water potential and disrupting membranes and cell processes such as photosynthesis. The timing, intensity, and duration of drought spells determine the magnitude of the effect of drought.

37.5
Abiotic Stress due to Freezing Temperature

Freezing temperature greatly limits the geographical distribution of cultivated plants and often causes severe losses in agriculture production. Biotechnology offers new strategies that can be used to develop transgenic crop plants with improved tolerance to freezing stress. A number of genes have been isolated and characterized that are responsive to freezing stress. The designed genotype should be better than the available ones and must reach the farmers. Transgenic technologies have opened up many exciting possibilities to improve products with added value with application in food, agriculture, animal husbandry, environment, medicine and industry. It also offers uncommon opportunities for improvement in genetic potential of plants and animals by introduction or removal of gene or genes that regulate a specific trait.

The conventional breeding methods complemented by an array of biotechnological tools can be used to augment vegetable production by saving time and resources. Molecular and genomic analyses have facilitated gene discovery and enabled genetic engineering using several functional or regulatory genes to activate specific or broad pathways related to abiotic stress tolerance in plants [18]. The potential outcome can be in the form of development of specific vegetable varieties that are more resistant to biotic and abiotic stresses, enhanced nutritional level of food items, enhanced shelf life of perishable farm produce, conversion of organic waste into biofuels.

37.6
Abiotic Stress-Tolerant Variety Development in Vegetables

AVRDC – The World Vegetable Center has developed tomatoes and Chinese cabbage with general adaptation to hot and humid tropical environments and low-input cropping systems since the early 1970s. This has been achieved by developing heat-tolerant and disease-resistant breeding lines. The Center has made significant contributions to the development of heat-tolerant tomato and Chinese cabbage lines and the subsequent release of adapted, tropical varieties worldwide. The key to achieving high yields with heat-tolerant cultivars is the broadening of their genetic base through crosses between heat-tolerant tropical lines and disease-resistant temperate or winter varieties [19]. The heat-tolerant tomato lines were developed using heat-tolerant breeding lines and landraces from the Philippines (e.g., VC11-3-1-8, VC 11-2-5, Divisoria-2) and the United States (e.g., Tamu Chico III, PI289309) [20]. However, lower yields in the heat-tolerant lines are still a concern.

More heat-tolerant varieties are required to meet the needs of a changing climate, and these must be able to match the yields of conventional, nonheat-tolerant varieties under nonstress conditions. A wider range of genotypic variation must be explored to identify additional sources of heat tolerance. An AVRDC – The World Vegetable Center breeding line, CL5915, has demonstrated high levels of heat tolerance in Southeast Asia and the Pacific. The fruit set of CL5915 ranges from 15–30% while there is complete absence of fruit set in heat-sensitive lines in mean field temperatures of 35 °C. Genetic studies at AVRDC – The World Vegetable Center indicate that heat tolerance in CL5915, based on fruit set and fruit number per cluster, is controlled by additive and dominant effects [21]. However, bimodality of fruit set distribution and recovery of tolerant lines in early backcross generations suggests that only a few major genes and modifiers may control the heat tolerance trait [22, 23]. Since then new breeding lines have been developed from CL5915 and other sources that exhibit increased heat tolerance. In Egypt, CL5915 lines were best combiners for percentage fruit set and total yield in hybrids developed from heat-tolerant and heat-sensitive lines [24].

Germplasm evaluation for heat tolerance at AVRDC – The World Vegetable Center conventionally relied upon field screening during the hot and humid season, with measurement of fruit set and yield. Generally, less than 1% of the screened lines or accessions exhibit a high level of heat tolerance [25]. Although field screening is

effective, the accuracy and speed of the process could be improved through the use of molecular markers.

Unfortunately, using classical breeding to select plants with greater resistance to abiotic stresses is inhibited by the difficulty of working with a trait that is controlled by several genes [3]. Since there are similar physiological changes that occur in drought and freezing, the types of genes that are expressed in the plants defense are similar [26]. Fortunately, the molecular biology behind the gene expression that provides cold acclimation in plants is being characterized more comprehensively [26]. Studies on cold-regulated gene expression in *Arabidopsis* have shown that many of the genes are regulated by the *CBF/DREB1* transcription factors (TFs). It is believed that the COR proteins that are regulated by *CBF*/DREB1 increase the production of membrane-stabilizing proteins and sugars [27]. Using genes that code for transcription factors is a promising method for genetic engineering because many of the ways in which plants can adapt to cold, drought, oxidative stress, and extreme temperatures is through transcriptional control [4, 28]. Transcription factors that are a part of the regulon to help prevent the effects of abiotic stress have been constitutively overexpressed to promote a greater amount of tolerance. Many of the studies in commercial crops were based upon *Arabidopsis* because not only the structural proteins but also the entire stress tolerance regulons are conserved, making it a valuable model for biotechnological research [28].

The purpose of this chapter is to provide a compendium of TFs related to the abiotic stress and development of transgenic crops that have been modified to reduce the effects of abiotic stress. All of the crops overexpress a gene that codes for a transcription factor that will activate a gene and a stress response to re-establish cellular homeostasis [4]. It is important that stress responses to abiotic factors are studied because it will help take further the study of functional genomics. The use of transcription factors discussed here in genetic engineering could be a vital feature of agricultural biotechnology.

37.7
Role of Transcription Factors in Plant Stress Tolerance

Plant stress responses are regulated by multiple signaling pathways that activate gene transcription and its downstream machinery. Plant genomes contain a large number of transcription factors; for example, *Arabidopsis* dedicates about 5.9% of its genome coding for more than 1500 TFs [29]. Most of these TFs belong to a few large multigene families, for example, MYB, AP2/EREBP, bZIP, and WRKY. Individual members of the same family often respond differently to various stress stimuli; on the other hand, some stress-responsive genes may share the same TFs, as indicated by the significant overlap of the gene expression profiles that are induced in response to different stresses [30–34]. Some key examples are discussed below.

The dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) bind to DRE and CRT *cis*-acting elements that contain the same motif (CCGAC). Members of the CBF/DREB1 family, such as CBF1, CBF2, and CBF3 (or

DREB1B, DREB1C, and DREB1A, respectively) are themselves stress inducible. DREB/CBF proteins are encoded by AP2/EREBP multigene families and mediate the transcription of several genes such as rd29A, rd17, cor6.6, cor15a, erd10, kin1, kin2, and others in response to cold and water stress [31, 35–39].

A significant improvement in stress tolerance was found upon overexpression of a single TF in engineered *Arabidopsis thaliana* plants. *Arabidopsis* cold acclimation is associated with the induction of COR (cold-regulated) genes by the CRT/DRE *cis*-regulatory elements [40]. Jaglo-Ottosen *et al*. [41] showed that increased expression of *Arabidopsis* CBF1 induces the expression of the cold-regulated genes cor6.6, cor15a, cor47, and cor78, and increased the freezing tolerance of nonacclimated *Arabidopsis* plants. *Arabidopsis* transformation with the DREB1A gene [42] driven either by the strong constitutive promoter of the cauliflower mosaic virus (35SCaMV) or by a DRE-containing promoter from the dehydration-induced gene (rd29A) resulted in a marked increase in tolerance to freezing, water, and salinity stress. Similar to the CBF1 transgene, constitutive expression of DREB1A transcription factor resulted in an increased expression of its downstream targeted genes, such as rd29A, rd17, cor6.6, cor15a, erd10, and kin1. Overexpression of CBF3 in *Arabidopsis* also increased freezing tolerance and, more interestingly, resulted in multiple biochemical changes associated with cold acclimation: elevated levels of proline and total soluble sugars, including sucrose, raffinose, glucose, and fructose [43]. Plants overexpressing CBF3 also had elevated D1-pyrroline- 5-carboxylate synthetase (P5CS) transcript levels, suggesting that the increase in proline levels had resulted, in part, from increased expression of the key proline biosynthetic enzyme P5CS.

Components of the *Arabidopsis* CBF/DREB cold response pathway were also found in *Brassica napus* and other plant species [44]. Constitutive overexpression of the *Arabidopsis* CBF genes in transgenic *B. napus* plants induced expression of orthologues of *Arabidopsis* CBF-targeted genes and increased the freezing tolerance of both nonacclimated and cold-acclimated plants. Recently, expression of *Arabidopsis* CBF1 in tomato plants has been shown to confer elevated tolerance to chilling and oxidative stress [45]. However, the expression of COR genes was not induced, while reactive oxygen species (ROS) scavenger genes, for example, CAT1, were activated. Recently, a close CBF/DREB1 homologue, CBF4, was isolated in *Arabidopsis*. The expression of CBF4 is rapidly induced during drought stress and by abscisic aid (ABA) treatment, but not by cold, thereby distinguishing it from CBF/DREB1 transcription factors. Overexpression of CBF4 under the constitutive CaMV35S promoter resulted in the expression of cold- and drought-induced genes under nonstress conditions, and the transgenic *Arabidopsis* plants showed more tolerance to freezing and drought conditions [46].

ABA signaling plays a vital role in plant stress responses as evidenced by the fact that many of the drought-inducible genes studied to date are also induced by ABA. Two TF families, bZIP and MYB, are involved in ABA signaling and its gene activation. Many ABA-inducible genes share the (C/T) ACGTGGC consensus, *cis*-acting ABA-responsive element (ABRE) in their promoter regions [47, 48]. Several ABRE binding proteins, including rice TRAB and *Arabidopsis* AREB/ABF and ABI5, which interact with ABRE and regulate gene expression, have been isolated [49–53].

Recently, Abe *et al.* [55] showed that the *Arabidopsis* MYB transcription factor proteins, AtMYC2 and AtMYB2, function as transcriptional activators in ABA-inducible gene expression, suggesting a novel regulatory system for gene expression in response to ABA, other than the ABRE-bZIP regulatory system.

Constitutive expression of ABF3 or ABF4 demonstrated enhanced drought tolerance in *Arabidopsis* plants, with altered expression of ABA/stress-responsive genes, for example, rd29B, rab18, ABI1, and ABI2 [54]. Several ABA-associated phenotypes, such as ABA hypersensitivity and sugar hypersensitivity, were observed in transgenic plants. Moreover, salt hypersensitivity was observed in ABF3- and ABF4-overexpressing plants at the germination and young seedling stage, indicating the possible participation of ABF3 and ABF4 in the salt response at these particular developmental stages. Improved osmotic stress tolerance in 35S:AtMYC2/AtMYB2 transgenic plants, as judged by an electrolyte leakage test [55], is yet another example of how plant engineering with transcriptional activators of ABA signaling can provide a means of improving plant stress tolerance.

37.8
Utilizing Heat Stress Transcription Factors to Increase Heat Stress Tolerance

Heat is another abiotic stress that can affect the way a plant develops, even as a seedling. Heat stress transcription factors (*HSF*) are expressed to induce the production of chaperones used to protect proteins from forming negative physiological interactions, such as conformational change in protein structure [56, 57]. This function of *HSF*s can be used to increase longevity under heat stress by introducing genes that code for *HSF*s in one plant and overexpress them either in the same plant or in a different species [58]. The sunflower gene *HaHSFA9* has recently been transferred into tobacco with a *DS10* promoter [58]. The *DS10* promoter was used because it provides two orders of magnitude greater expression than a *35s* promoter without the common adverse effects such as decreased yield and size, and seed specificity [58]. Modifying tobacco with *DS10:HaHSFA9* from sunflower gave its seeds the ability to overexpress the production of *HSP*s, thereby increasing tolerance to heat stress [58]. Seven days after the heat treatment at 50 °C a handful of the controls germinated (0–6%), whereas $24 \pm 5\%$ of the DS10:Ha*HSF*A9-transformed plants germinated [58]. It is important to note that because the *HSF*s are conserved among many other plants, future experiments can be formulated to provide seed heat stress/deterioration resistance using orthologous factors [58].

Similar to osmotic stress, the heat shock response is primarily regulated at the transcriptional level. Thermoinducibility is attributed to conserve *cis*-regulatory promoter elements (HSEs) that are the binding sites for the *trans*-active heat shock factors [59]. The HSEs share a common consensus sequence "nGAAnnTTCnnGAAn." Plant HSFs, which are further categorized into three classes, A, B, and C, appear to be a unique family containing a number of members: 21 from *Arabidopsis*, more than 16 from tomato, and 15 from soybean [60]. Hsps are chaperones, which function during both normal cell growth and stress conditions;

therefore, it is not surprising that HSFs provide diverse functions that differentially control the activation of heat shock genes [59, 61, 62]. It has been shown that overexpression of HSF1 and HSF3 (class A) leads to the expression of several hsp genes conferring thermotolerance in transgenic plants [63, 64]. In tomato plants, overexpression of HsfA1 resulted in heat stress tolerance, while HsfA1 antisense plants and fruits were extremely sensitive to elevated temperatures [62]. Analysis of the transgenic plants disclosed that HsfA1 has a unique role as a master regulator for the synthesis of other HSFs such as HSFs A2 and B1 as well as Hsps. HSFs may also play a role in controlling cell death. The rice spl (spotted leaf) gene spl7 encodes a class-A HSF and the spl7 transgenic rice showed no lesions (spotted leaf) or delay in development of lesions [65]. The experiment suggested that spl7 might participate in controlling cell death that is caused by environmental stresses such as high temperature.

These studies demonstrate the important role of TFs in the acquisition of stress tolerance, which may ultimately contribute to agricultural and environmental practices. Although plant transformation with stress-responsive TFs permits over-expression of downstream stress-associated multiple genes, it may also activate additional nonstress genes that adversely affect the normal agronomic characteristics of a crop. One common negative effect of TF-modified plants is the growth retardation in transgenic plants that constitutively express TFs [42, 45, 54, 55]. For example, a positive correlation was found between the levels of DREB1A expression, the level of expression of the target gene RD29A, and the degree to which plants growth is stunted [38]. These negative effects can be partially prevented by the use of stress-inducible promoters that control the expression of the TF [42].

37.9
Other TFs Used to Increase Stress Tolerance

It has recently been determined that *Oryza sativa* can become more stress tolerant of drought and salt at the vegetative stage by transforming the plant with *SNAC11* and a CaMV 35s promoter [66]. In addition, there was 17.2–24.0% greater fertility (seed number) in the transformed rice than the negative control after 5 days of severe and moderate salt stress. In addition to phenotypic results, the expression levels of the *SNAC11* gene were determined using a Northern blot [66]. It was possible to determine the localization of expression of *SNAC11* by incorporating GFP in the construct. The expression levels in the transgenic plants were located mainly in the leaves where there was curling, which helped in closing stomata and inhibiting water loss [66]. Another novel gene that controls a transcription factor inhibits the effects of cold, salt, and drought stress in rice is *OsCOIN* (*O. sativa* cold inducible) [67]. By controlling the gene with a CaMV35s promoter and transforming the rice with *Agrobacterium*, it was possible to increase tolerance to cold, salt, and drought stress [67]. Moreover, 2 week-old rice was exposed to a temperature of 4 °C for 60, 71, and 84 h and allowed to recover for 2 weeks where it was found that a significantly greater percentage of *OsCOIN* transgenic rice grew than the wild

type [67]. In addition, the results from a 20 day-long salt stress test showed that *OsCOIN* transgenic rice germinated 35% faster and taller than the wild-type rice [67]. Also, it was determined that proline concentration in the cell helped determine *OsCOIN*'s ability to function [67].

Lettuce (*Latuca sativa* L.), one of the most popularly grown vegetables, worldwide, is limited in productivity by its own ability to fight off the effects of drought and cold temperatures [68]. Recently, lettuce has been successfully transformed with *Arabidopsis* ABA-responsive binding factor3 (ABF3) gene so the crop can overexpress the genes necessary to tolerate drought and cold [69]. Again, using *Agrobacterium*-mediated transformation, a construct was obtained containing not only the gene of interest, ABF3 (under maize ubiquitin promoter), but also gfp gus and the selectable marker hph under a 35S promoter [69]. ABF3 can be used to improve lettuce tolerance as a seedling and an adult, as the wild-type lines were significantly more vulnerable to the stresses used [69]. The Southern blotting was performed by digesting genomic DNA with *HindIII* and probing with ^{32}P *ABF3*. This study determined the inheritance of the T1 progeny to be 3: 1 in transgenic lines with a single copy of the *ABF3* [69].

Plants resist water or drought stress in many ways. In slowly developing water deficit, plants may escape drought stress by shortening their life cycle [70]. However, the oxidative stress of rapid dehydration is very damaging to the photosynthetic processes, and the capacity for energy dissipation and metabolic protection against reactive oxygen species is the key to survival under drought conditions [70, 71]. Tissue tolerance to severe dehydration is not common in crop plants but is found in species native to extremely dry environments [35]. Genetic variability for drought tolerance in *Solanum lycopersicum* is limited and inadequate. The best sources of resistance are other species in the genus *Solanum*. The Tomato Genetics Resource Center (TGRC) at the University of California, Davis, has assembled a set of the putatively stress-tolerant tomato germplasm that includes accessions of *S. cheesmanii*, *S. chilense*, *S. lycopersicum*, *S. lycopersicum* var. *cerasiforme*, *S. pennellii*, *S. peruvianum*, and *S. pimpinellifolium*. *S. chilense* and *S. pennelli* are indigenous to arid and semiarid environments of South America. Both species produce small green fruit and have an indeterminate growth habit. *S chilense* is adapted to desert areas of Northern Chile and often found in areas where no other vegetation grows [72, 73], has finely divided leaves and well-developed root system [74], and has a longer primary root and more extensive secondary root system than cultivated tomato [75]. Drought tests show that *S. chilense* is five times more tolerant of wilting than cultivated tomato. *S. pennellii* has the ability to increase its water use efficiency under drought conditions unlike the cultivated *S. lycopersicum* [75]. It has thick, round waxy leaves, is known to produce acyl sugars in its trichomes, and its leaves are able to take up dew [72].

Transfer and utilization of genes from these drought-resistant species will enhance tolerance of tomato cultivars to dry conditions, although wide crosses with *S. pennellii* produce fertile progenies, *S. chilense* is cross-incompatible with *S. lycopersicum* and embryo rescue through tissue culture is required to produce progeny plants. Research at AVRDC – The World Vegetable Center and other institutions is in

progress to identify the genetic factors underlying drought tolerance in *S. chilense* and *S. pennellii*, and to transfer these factors into cultivated tomatoes.

Related wild tomato species have shown strong salinity tolerance and are sources of genes as coastal areas are common habitat of some wild species. Studies have identified potential sources of resistance in the wild tomato species, *S. cheesmanii*, *S. peruvianum*, *S pennelii*, *S. pimpinellifolium*, and *S. habrochaites* [76–78]. Attempts to transfer quantitative trait loci (QTL) and elucidate the genetics of salt tolerance have been made using populations involving wild species. Elucidation of mechanism of salt tolerance at different growth periods and the introgression of salinity tolerance genes into vegetables would accelerate development of varieties that are able to withstand high or variable levels of salinity compatible with different production environments.

37.10
A Review of Abiotic Stress Tolerance in Chili Pepper

Pepper is a member of the family Solanaceae, which is one of the largest families in the plant kingdom and includes more than 3000 species [79]. The Solanaceae family includes important crops, such as pepper, tomato, tobacco, potato, and eggplant and has been widely cultivated over the years for human nutrition and health. *Capsicum* species are consumed worldwide and are valued because of their unique color, pungency, and aroma. *Capsicum* peppers include *Capsicum annuum*, *C. chinense*, *C. baccatum*, *C. frutescens*, and *C. pubescens* and are cultivated in different parts of the world. Of these, the varieties of the chili pepper plant species *C. annuum*, having a modest size diploid genome ($2n = 24$), are the most heavily consumed due to their nutritional value and spicy taste [80]. The chemical that is primarily responsible for the pungency of *C. annuum* has been identified as capsaicin [81], which elicits numerous biological effects and is the target of extensive investigation. The pepper production is influenced by many abiotic factors such as high temperature and salinity. In pepper, high-temperature exposure at the preanthesis stage did not affect pistil or stamen viability, but high postpollination temperatures inhibited fruit set, suggesting that fertilization is sensitive to high-temperature stress. In pepper, salt stress significantly decreases germination, shoot height, root length, fresh and dry weight, and yield. Yildirim and Guvenc [82] reported that pepper genotypes Demre, Ilica 250, 11-B-14, Bagci Carliston, Mini Aci Sivri, Yalova Carliston, and Yaglik 28 can be useful as sources of genes to develop pepper cultivars with improved germination under salt stress. In Tunisia, the root system of salt-tolerant cultivar "Beldi" was unaffected by salt stress. In addition, "Beldi" significantly out-yielded other test cultivars at high salt treatments.

Hong *et al.* [83] reported the isolation of pepper GDSL-type lipase (*CaGLIP1*) gene and functionally characterized, from pepper leaf tissues infected by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). GDSL-type lipase is a hydrolytic enzyme whose amino acid sequence contains a pentapeptide motif (Gly-X-Ser-X-Gly) with active serine (Ser). The *CaGLIP1* gene was preferentially expressed in pepper leaves during the

compatible interaction with *Xcv*. Treatment with salicylic acid, ethylene, and methyl jasmonate induced *CaGLIP1* gene expression in pepper leaves. Dehydration and wounding mediated by sodium nitroprusside, methyl viologen, high salt, and mannitol also induced early and transient *CaGLIP1* expression in pepper leaf tissues. Virus-induced gene silencing of *CaGLIP1* in pepper conferred enhanced resistance to *Xcv*, accompanied by the suppressed expression of basic PR1 (*CaBPR1*) and defensin (*CaDEF1*) genes. During seed germination and plant growth, the *CaGLIP1* transgenic plants showed drought tolerance and differential expression of drought- and ABA-inducible genes *AtRD29A*, *AtADH*, and *AtRab18*. ABA treatment differentially regulated seed germination and gene expression in wild-type and *CaGLIP1* transgenic *Arabidopsis*. Overexpression of *CaGLIP1* also regulated glucose- and oxidative stress signaling. These results indicated that CaGLIP1 modulates disease susceptibility and abiotic stress tolerance.

A gene encoding a putative guanosine 5′-diphosphate (or 5′-triphosphate) 3′-diphosphate ((p)ppGpp) synthetase, designated PepRSH (*Pep*per *RelA*/*SpoT* homologue), was isolated from hot peppers by Kim *et al*. [84]. *PepRSH* was found to contain 5 introns and 6 exons and a 2166-bp open reading frame encoding a protein of 721 amino acids; this protein displayed significant homology to other plant. (p)ppGpp synthetase *PepRSH* transcripts were highly accumulated in nonhost resistance response-induced leaves and in leaves following induction with salicylic acid, methyl jasmonate, wounding, hydrogen peroxide, and ultraviolet B. The expression of *PepRSH* was also influenced by abiotic stresses, such as flooding and high salinity. The deduced PepRSH protein has a putative chloroplast-targeting transit peptide at its N-terminus, and immunolocalization studies verified the translocation of PepRSH to the chloroplast. The predicted PepRSH protein is markedly similar to known plant and bacterial RSH proteins. Expression of a putative (p)ppGpp synthetase domain in an *Escherichia coli* single mutant ($RelA^{-}SpoT^{+}$) complemented growth of the mutant, but not of an *E. coli* double mutant ($RelA^{-}SpoT^{-}$), demonstrating that PepRSH has (p)ppGpp synthetase activity only in the (p)ppGpp synthetase domain. Site-directed mutagenesis of the conserved histidine and aspartic acid (HD) site in the putative HD domain of PepRSH revealed that the histidine and aspartic acid dual sites were critical residues for the (p)ppGpp synthetase activity of PepRSH protein. Mutation of the HD site limited the tolerance of bacteria to both salt and osmotic stress. Their results indicate that pepper plants have a (p)ppGpp regulatory system that is similar to that of bacteria and that may transduce stress-related signals through the regulation of (p)ppGpp by PepRSH localized in chloroplasts.

Chung *et al*. [85] experimented with full-length Capsicum annuum calcium-dependent protein kinase 3 (CaCDPK3). cDNA clone was selected from the chili pepper expressed sequence tag database. CaCDPK3 belongs to a multigene family in the pepper genome gene was rapidly induced in response to various osmotic stress factors and exogenous abscisic acid application in pepper leaves. Moreover, CaCDPK3 RNA expression was induced by an incompatible pathogen and by plant defense-related chemicals such as ethephon, salicylic acid, and jasmonic acid. The biochemical properties of a CDPK3 were investigated using a CaCDPK3 and

glutathione *S*-transferase (GST) fusion protein. The recombinant proteins retained calcium binding ability and displayed autophosphorylation activity *in vitro* in a calcium-dependent manner. Furthermore, transient expression studies showed that CaCDPK3 fused with soluble modified green fluorescent protein (smGFP) localized to the cytosol in chili pepper protoplasts. The CaCDPK3 was implicated in biotic and abiotic stresses in pepper plants.

Cho *et al*. [86] isolated three different cDNA clones (pCaXTH1, pCaXTH2, and pCaXTH3) from water-stressed hot pepper that encode XTH (Xyloglucan endo-transglucosylase/hydrolase that has been recognized as a cell wall-modifying enzyme, participating in the diverse physiological roles) homologues. RT-PCR analysis showed that three CaXTH mRNAs were concomitantly induced by a broad spectrum of abiotic stresses, including drought, high salinity, and cold temperature, and in response to stress hormone ethylene, suggesting their role in the early events in the abiotic-related defense response. Transgenic *Arabidopsis* plants that constitutively expressed the CaXTH3 gene under the control of the CaMV 35S promoter exhibited abnormal leaf morphology; the transgenic leaves showed variable degrees of twisting and bending along the edges, resulting in a severely wrinkled leaf shape. Microscopic analysis showed that 35S-CaXTH3 leaves had increased numbers of small-sized cells, resulting in disordered, highly populated mesophyll cells in each dorsoventral layer, and appeared to contain a limited amount of starch. In addition, the 35S-CaXTH3 transgenic plants displayed markedly improved tolerance to severe water deficit and to a lesser extent to high salinity in comparison to the wild-type plants. These results indicate that CaXTH3 is functional in heterologous *Arabidopsis* cells, thereby effectively altering both the cell growth and the response to abiotic stresses. Although the physiological function of CaXTHs is not yet clear, there are several possibilities for their involvement in a subset of physiological responses to counteract dehydration and high-salinity stresses in transgenic *Arabidopsis* plants.

An *et al*. [87] isolated and functionally characterized the pepper (*C. annuum* L.) gene *CaPMEI1*, which encodes a pectin methylesterase inhibitor protein (PMEI), in pepper leaves infected by *X. campestris* pv. *vesicatoria* (*Xcv*). *CaPMEI1* transcripts are localized in the xylem of vascular bundles in leaf tissues, and pathogens and abiotic stresses can induce diVerential expression of this gene. PuriWed recombinant CaPMEI1 protein not only inhibits PME but also exhibits antifungal activity against some plant pathogenic fungi. Virus-induced gene silencing of *CaPMEI1* in pepper confers enhanced susceptibility to *Xcv*, accompanied by suppressed expression of some defense-related genes. Transgenic *Arabidopsis CaPMEI1* overexpression lines exhibit enhanced resistance to *Pseudomonas syringae* pv. *tomato*, mannitol, and methyl viologen, but not to the biotrophic pathogen *Hyaloperonospora parasitica*. Together, their results suggest that *CaPMEI1*, an antifungal protein, may be involved both in basal disease resistance and in drought and oxidative stress tolerance in plants.

A full-length cDNA clone of the *Capsicum annuum* ankyrin-repeat domain C3H1 zinc finger protein (CaKR1) was identified in a chili pepper by Seong [88] and his group using the expressed sequence tag (EST) database. The deduced amino acid sequence of CaKR1 showed a significant sequence similarity (46%) to the ankyrin-repeat protein in very diverse family of proteins of *Arabidopsis*. The gene was induced

in response to various biotic and abiotic stresses in the pepper leaves, such as salicylic acid (SA) and ethephon, as well as by an incompatible pathogen. CaKR1 expression was highest in the root and flower, and its expression was induced by treatment with agents such as NaCl and methyl viologen, as well as by cold stresses. These results showed that CaKR1 fusion with soluble, modified green fluorescent protein (smGFP) was localized to the cytosol in *Arabidopsis* protoplasts, suggesting that CaKR1 might be involved in responses to both biotic and abiotic stresses in pepper plants.

The same research group of Seong [89] reported the full-length cDNA of *CaAbsi1* from pepper encodes a presumptive protein of 134 amino acid residues that has homology to a putative zinc finger protein in its C-terminus. The deduced amino acid sequence has 50% homology to *O. sativa* NP001049-274, the function of which is unknown. Expression of *CaAbsi1* was reduced in response to inoculation of nonhost pathogens. On the other hand, it was induced 1 h after exposure to high concentrations of NaCl or mannitol, and 6 h after transfer to low temperature. Induction also occurred in response to oxidative stress, methyl viologen, hydrogen peroxide, and abscisic acid. Their results suggest that *CaAbsi1* plays a role in multiple responses to wounding and abiotic stresses.

Choi *et al.* [90] isolated and functionally characterized the pathogen-responsive CaM gene, *Capsicum annuum* calmodulin 1 (*CaCaM1*), from pepper (*C. annuum*) plants. The cellular function of *CaCaM1* was verified by *Agrobacterium* spp.-mediated transient expression in pepper and transgenic overexpression in *A. thaliana*. *Agrobacterium* spp.-mediated transient expression of *CaCaM1* activated ROS, nitric oxide (NO) generation, and hypersensitive response (HR)-like cell death in pepper leaves, ultimately leading to local acquired resistance to *X. campestris* pv. *vesicatoria*. *CaCaM1*-overexpression (OX) *Arabidopsis* exhibited enhanced resistance to *P. syringae* and *H. parasitica*, which was accompanied by enhanced ROS and NO generation and HR-like cell death. Treatment with the calcium channel blocker suppressed the oxidative and NO bursts and HR-like cell death that were triggered by*CaCaM1* expression in pepper and *Arabidopsis*, suggesting that calcium influx is required for the activation of *CaCaM1*-mediated defense responses in plants. Upon treatment with the CaM antagonist, virulent *P. syringae* pv. *tomato*-induced NO generation was also compromised in *CaCaM1*-OX leaves. Together, these results suggest that the *CaCaM1* gene functions in ROS and NO generation are essential for cell death and defense responses in plants.

37.11
Transgenic Chili Pepper for Abiotic Stress Tolerance

Chatzidimitriadou *et al.* [91] reported the stress tolerance and regeneration capability of transgenic pepper plants carrying a *sod* gene, encoding a tomato chloroplast-localized Cu/Zn SOD protein. The expression of the sod gene was confirmed by enzymatic staining following polyacrylamide gel electrophoresis (PAGE), revealing a "novel" band, which could represent a heterodimeric enzyme. Transgenic T1 and T2 progeny plants were exposed to different oxidative stresses, including Methyl

viologen (MV), and drought and found to have an increased resistance to oxidative damage. Furthermore, the SOD-carrying transgenic pepper plants showed increased levels of regeneration efficiency compared to the wild-type pepper plants. Pepper is a recalcitrant species in terms of its *in vitro* regeneration ability, but it could be extremely useful for the development of pharmaceuticals. Their approach enables the extent of the use of pepper for genetic transformation and the production of high-value products in plants particularly the large fruit varieties.

37.12
Abiotic Stress Tolerance in Onion (*Allium cepa L.*)

The genus *Allium* is an important member of onion family (Alliaceae; subfamily Allioideae) [92]. Alliums represent a major group among bulbous crops and these have been cultivated for food and medicine or religious purposes. They are consumed raw or cooked as a vegetable or as a pickle and also as dehydrated product in the form of powder, flakes, or bulbs [93, 94]. Most of these crops are strongly flavored and are characterized by sulfur-containing compounds, which impart them their distinctive smell and pungency.

Onion has the primary center of origin in central Asia and secondary center in the Near East. It is an important commercial vegetable crop grown worldwide. In terms of global weight of vegetables produced, at nearly 28 million ton per annum, only tomatoes and cabbage exceed bulb onions in importance [95]. In terms of area, India ranks first in the world with over 480 000 ha accounting for around 21% of the world area under onion cultivation. Globally, the country occupies the second position after China in onion production with a production share of around 14%. Besides India and China, the other major onion producing countries are Turkey, Pakistan, Brazil, the United States of America, Iran, Spain, and Japan. India accounts for more than 70% share in the export of fresh onions. Productivity, however, is at around 11.4 million ton ha^{-1}, which is lower than the world average of 17.3 million ton ha^{-1}. In India, onion is extensively cultivated over a large area spread almost throughout the country. Despite the development of impressive irrigation potential, which ensured food security to India during the past three decades, agriculture in India is still considerably affected by climatic variability. Shortage of onions and potatoes in 1998 and gluts of onions, potatoes, rice, and wheat in 2000 in India were largely due to variable climatic conditions [96]. The commonly cultivated alliums and their areas of cultivation in India are presented in Table 37.1.

Abiotic stress includes drought, heat, flood, salinity, mineral deficiency, toxicity, and chilling or freezing stress. The nature and magnitude of stress vary. These stresses are linked with natural phenomenon and their scale varies at temporal and spatial dimensions. There is hardly a landmass in the world that is not influenced by one or the other of these stress factors. Drought, salinity, extreme temperatures, and oxidative stress are often interconnected and may induce similar cellular damage. They are very complex stimuli that possess many different yet related attributes, each of which may provide the plant cell with quite different information. These abiotic

Table 37.1 Cultivated alliums and their areas of cultivation in India.

Common name	Botanical name	Chromosome number (2n)	Areas of cultivation
Common onion	A. *cepa* var. *cepa* L.	16	Throughout India
Shallot	*A. cepa* var. *aggregatum* G. Don	16	Himalayas, Peninsular India
Welsh onion	*A. fistulosum* L.	16	Western Himalayas, Northeast region
Chinese chives	*A. tuberosum* Rottl. ex Spreng.	32	Western Himalayas Northeast region
Garlic	*A. sativum* L.	16	Throughout India
Leek	*A. ampeloprasum* var. porrum (L.) J. Gay	32	Western Himalayas
Chives	*A. schoenoprasum* L.	16, 24, 32	Western Himalayas

factors lead to a series of morphological, physiological, biochemical, and molecular changes that adversely affect growth and productivity of crop plants [97]. For example, low temperature may immediately result in mechanical constraints, changes in the activities of macromolecules, and reduced osmotic potential in the cellular milieu [98]. Onion is a shallow-rooted crop plant that is predominantly grown under rain-fed conditions and is, therefore, exposed to frequent droughts during its ontogeny. There are no studies available on abiotic stress-tolerant onion. Also, no single germplasm/variety or hybrid of onion has been developed/released for cultivation in those regions affected by above-mentioned stresses. Few workers have studied the effect of fructans, polyamines, and glutathione *S*-transferases on abiotic tolerance in onion, which are elaborated here.

37.13 Role of Fructans in Freezing and Drought Tolerance

Sucrose and starch are the primary vegetative storage carbohydrates in tropical and subtropical grasses, while temperate and cool zone grasses mainly accumulate fructose polymers called fructans. Numerous studies have been published that attempted to correlate fructan concentrations with freezing and drought tolerance. Fructan is considered a short-term storage carbohydrate that has been implicated for many years in stress tolerance mechanisms in plants. Fructans (polyfructosylsucrose) consist of polymers of Fru attached to Suc and serve as an important storage carbohydrate in approximately 15% of flowering plant species [99]. The fructan residues are either linked by a (2–1) b-d-glycosidic bond, as in inulin derived from *Cichorium intybus* L. [100], or by a (2–6) b-d-glycosidic bond, as in levans (e.g., *Phleum pratense* L. [101]). In most grasses, branched fructans containing both types of linkages are produced (e.g., *Triticum aestivum* L. [102]). The effect of fructans on

Table 37.2 Five types of fructan in plants showing a representative plant species in which the respective type of fructan has been identified.

Type	Representative species	Linkage (β)	Initial trisaccharide
Inulin	Chicory, Jerusalem artichoke	2-1	1-ketose
Levan	*Dactylis glomerata*	2–6	6-ketose
Branched	Wheat, barley	2-1 and 2-6	1- and 6-ketose
Inulin neoseries	Onion, asparagus, Lolium	2-1	6G-kestotriose (neokestose)
Levan neoseries	Lolium, oats	2-6	6G-kestotriose (neokestose)

liposomes has indicated that a direct interaction between membranes and fructans is possible. This helps to prevent leakage when water is removed from the system either during freezing or during drought. Five types of fructans have been identified in plants (Table 37.2).

In Liliaceae, such as onion and asparagus, a different type of inulin is present, namely, the inulin neoseries. These are linear with 2–1 linkages but with the glucose molecule between two fructosyl subunits [103]. Fructan synthesis is initiated when photosynthesis exceeds demand, reportedly when sucrose levels in sink organs reach a critical level [104]. Synthesis is complex due to differences between species in linkages, branching patterns, and sizes; however, a model for synthesis in plants that includes four fructosyltransferases has been proposed [105]. An enzyme capable of synthesizing 6G-kestose (neokestose) in onion was first described by Shiomi [103] and was cloned by Vijn *et al*. [106].

Soluble solids such as sucrose, glucose, fructose, and fructans are the primary nonstructural storage carbohydrates in onion [107, 108] that are used for regrowth of the plant in spring. Lower solid onions accumulate essentially no fructans and tend to be soft with low pungency, whereas onions with higher soluble solids tend to be firm and highly pungent. Fructans start to accumulate at the onset of bulbing, which coincides with increased fructosyltransferase activity [109, 110]. During growth, the fructan content of bulbs increases, but by the end of the growth season a decline in fructan content is observed [109]. Storage of onion bulbs leads to a further decrease in fructan levels and sprouting is induced [111]. This phenomenon severely reduces storage time for onion.

During cultivation, harvesting, handling, transportation, packaging, and storage, onion bulbs are exposed to different treatments, atmospheric conditions, and temperatures, all of which can affect their growth [112], their quality, and their physiological characteristics [113, 114]. The results of these effects could be responsible for several reactions and for stress causing important biochemical changes in the bulb tissues. Fructans are accumulated during the bulbing stage and then are catabolized during the regrowth and the sprout development of the bulbs [115]. They may have been implicated in protecting plants against water deficit by drought or low temperature, in inducing resistance to drought or cold stress, and in acting as osmoregulators [116]. Fructan accumulation during periods of reduced growth under

low but nonfreezing conditions (cold acclimation) has frequently been correlated with an increase in freezing tolerance [117, 118]. This correlation stimulated research to explain how fructan might be involved in protection from freezing stress.

37.14
Role of Polyamine and Abscisic Acid Interaction during Stress Tolerance

Polyamines (PAs), important growth regulatory polycationic molecules, have long been established to be involved in a wide range of plant growth and development processes such as embryogenesis, root development, flowering, tuber formation, senescence, and fruit ripening. They have also been implicated in plant responses to abiotic and biotic stresses [119, 120]. Commonly occurring PAs in higher plants include putrescine2^+ (Put), spermidine3^+ (Spd), and spermine4^+ (Spm) that are protonated at cytoplasmic pH. PAs appear to play a role in cell division, but not in cell elongation [121, 122]. Likewise, ABA regulates an array of plant processes and mediates plant responses to a variety of abiotic stresses [123]. ABA is also known to inhibit mitotic cell division [124, 125]. Since the levels of both PAs and ABA increase in plant tissues in response to abiotic stresses, a possibility of interaction between them in the regulation of plant processes including mitosis exists. The interaction between PAs and ABA in the regulation of mitosis and thereby growth is quite likely in view of the fact that both effectors are known to increase in concentration in plant tissues under abiotic stress conditions. Mahajan and Sharma [126] have reported such interactive effects of PAs and ABA on mitosis in terms of mitotic index and also measured catalase (CAT) activity to understand the involvement of redox metabolism in mediating the effects of PAs and/or ABA on mitosis in the root tips of onion. These interactions may be of significance for plant survival under stressful growth conditions. Since CAT decomposes H_2O_2, produced in higher concentrations in plant tissues in response to diverse abiotic stresses, altered CAT activities may be taken as changed cellular redox status.

37.15
Role of Antioxidants in Salt Stress in Onion

A majority of crop plants are relatively salt sensitive and are unable to tolerate low level of salinity [127]. Shanon [128] has also found a wide range of variability in salt tolerance between a number of agronomic species. Growth and yield of onion were affected when the irrigation water salinity exceeded 3 mS cm^{-1} [129]. Other investigators reported that high levels of salinity reduced onion vegetative growth, yield, and quality [130, 131].

Salt imposes several kinds of stresses upon plant. It causes drastic changes in the water potential, ion toxicity, ion imbalance, and oxidative stress [132–135]. Salt stress induces an oxidative stress via cellular accumulation of damaging active oxygen species (AOS) including superoxide radicals, hydrogen peroxide, and

hydroxyl radicals [136, 137]. AOS cause oxidative damage to different cellular components including membrane lipid, protein, and nucleic acid [138, 139]. Plants cope with this by producing enzymatic and nonenzymatic antioxidants [4, 140]. Among nonenzymatic antioxidants, glutathione has been indicated to scavenge oxygen species and improve seed germination and seedling growth under salt stress [141–143].

Glutathione is a tripeptide [α-glutamyle cysteinylglycine], which has been detected virtually in all cell compartments [142]. A wide range of biotic and abiotic factors induce plant GST expression. These include herbicides, heavy metal, pathogen attack, ethylene, ozone, plant growth factor auxin, salicylic acid, and hydrogen peroxide [144]. Environmental stresses such as osmotic stress [145], low temperature [146], saline stress [147], and cadmium [148] also induce GST expression in plant. Therefore, GSTs are thought to play vital roles in the diversity of stress physiologies through some other functions as well as GST-mediated detoxification. A protective role of these enzymes is suggested in the enzymatic upregulation of plant GSTs as being one of the important parameters under environmental conditions for cellular survival.

The GST activity in onion bulb has been enriched compared to other vegetable crops [149]. Onion bulb GSTs consist of five components including GSTa, GSTb, GSTc, GSTd, and GSTe [150]. Among these, GSTa and GSTb, with low activity, were termed as minor GSTs, and GSTc, GSTd, and GSTe, with high activity, were termed as dominant GSTs. In onion bulb, quercetin-4′-glucoside and quercetin-3,4′-diglucoside were also reported as physiological inhibitors of the dominant GSTs [150–152]. The 1-chloro- 2,4- dinitrobenzene (CDNB) conjugating activities of GSTc and GSTd, and to a lesser extent GSTe, were highly sensitive to the inhibitors, particularly quercetin-4′-glucoside. It has been reported that the activity level of the dominant GST, GSTe, was found to change over time of storage of onion bulb. The most studied endogenous substrate, anthocyanin that has been reported to have a positive correlation with GST accumulation, is also a strong inhibitor of the GSTs [153, 154]. Therefore, endogenous inhibitory substrates of onion GSTs might also affect the activity levels of the GSTs in onion bulb. Salama and Mutawa [155] studied the effect of NaCl on plasma membrane in the absence and presence of glutathione in onion. Glutathione ameliorated NaCl-induced plasma membrane changes and maintained its permeability and cell viability. The ameliorative effect of glutathione was more pronounced when added together with salt. The alleviating effect of glutathione might be through scavenging active oxygen species and hence lipid peroxidation produced under salt stress.

Although onion flavor intensity has been studied in response to various growing conditions, little is known about its response to salt stress. Improving the content of active compounds (volatile sulfur compounds, ascorbic acid, carbonyl compounds, vitamins, and flavonoids) is the most important pharmaceutical strategy and could be enhanced through different environmental stresses in some vegetables [156]. Moreover, salt stress may affect soluble solid content (SSC), bulb pungency as measured by total pyruvate (TPY), bulb sulfur (S) and sulfate (SO_4) accumulation, and flavor precursors and their biosynthetic intermediates in onion [157].

37.16
Genetic Transformation in Onion

Genetic engineering and *in vitro* regeneration protocols are two collaborative equipment to complement conventional *Allium* breeding and to develop high yield, biotic/abiotic stress resistant/tolerant cultivars, adapted to local ecological conditions [158–161]. Eady *et al.* [162] were the pioneer in *Agrobacterium tumefaciens*-mediated gene transformation and regeneration of *A. cepa*. Eady *et al.* [163] momentously achieved herbicide-resistant onion *A. cepa* and developed transgenics in *A. porrum* and *A. sativum* using *A. tumefaciens*-mediated transformation. Zheng *et al.* [164] followed similar methodology for development of a reproducible transformation system in *A. sativum* and production of transgenics resistant to beet armyworm (*Spodoptera exigua* Hubner). In a most recent experiment, transgenic chicory plants harboring the onion 6G-FFT under the control of the cauliflower mosaic virus 35S RNA promoter produced inulin of the neoseries in addition to linear inulin [106]. Sucrose Suc 1-fructosyltransferase (1-SST) is the key enzyme in plant fructan biosynthesis since it catalyzes *de novo* fructan synthesis from Suc. Vijn *et al.* [165] have cloned 1-SST from onion by screening a cDNA library using acid invertase from tulip as a probe. Expression assays in tobacco protoplasts showed the formation of 1-kestose from Suc.

Onion is an excellent system to study carbohydrate accumulation because recurrent selection has produced low and high fructan accumulating populations. For example, the onion population "Southport White Globe" was subjected to phenotypic recurrent selection for higher fructan content, shifting the population mean from 17 to >23%. Major QTL on chromosomes 5 and 8 are significantly (LOD > 3.5) associated with higher fructan concentrations.

37.17
Conclusions and Prospects

Plants have evolved mechanisms to respond at the morphological, anatomical, cellular, and molecular levels for avoidance of and/or tolerance to various abiotic stresses. In response to stress, plants respond by gene expression leading to cellular homeostasis and detoxification of toxins, ultimately aiming at recovery of growth. These adaptive mechanisms can be investigated by molecular, biochemical, and physiological studies. This chapter summarizes the recent efforts to improve abiotic stress tolerance in crop plants by employing some of the stress-related genes and transcription factors. Various transcription factors are involved in the regulation of stress-inducible genes. Functional genomic studies may provide tools for dissecting abiotic stress responses in plants through which networks of stress perception, signal transduction, and defense responses can be examined from transcriptomic through proteomic to metabolomic profiles of stressed tissues. A well-focused approach combining the molecular, physiological, and metabolic aspects of abiotic stress tolerance is required for bridging the knowledge gaps between the molecular or cellular expression of the genes and the whole-plant phenotype under stress.

There is a clear and urgent need to introduce stress tolerance genes into crop plants, in addition to establishing gene stacking or gene pyramiding. Transgenic research has opened up a new opportunity in crop improvement allowing the transfer of desirable gene(s) across species and genera for developing transgenic plants with novel traits, such as built-in protection, improved nutritional qualities, and so on. Efficient transformation system in Chili pepper and onion is a major limitation for developing transgenic plants. Although progress in improving stress tolerance has been slow, there are a number of reasons for optimism. The use of transgenes to improve the tolerance of crops to abiotic stresses remains an attractive option. Options targeting multiple gene regulation appear better than targeting single genes. This can be done by either combining multiple genes of a single protective pathway or by combining key regulatory genes of different protective pathways.

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References

1 Bray, E.A. (2002) *Plant Cell Environ.*, **25**, 153–161.
2 Capiati, D.A., País, S.M., and Téllez-Iñón, M.T. (2006) *J. Exp. Bot.*, **57**, 2391–2400.
3 Vinocur, B. and Altman, A. (2005) *Curr. Opin. Biotechnol.*, **16**, 123–132.
4 Wang, W., Vinocur, B., and Altman, A. (2003) *Planta*, **218**, 1–14.
5 Yamaguchi, T. and Blumwald, E. (2005) *Trends Plant Sci.*, **10** (12), 616–619.
6 Jones, R.A. (1986) *Acta Hortic.*, **190**, 101–114.
7 Cheeseman, J.M. (1988) *Plant Physiol.*, **87**, 57–550.
8 Bell, G.D., Halpert, M.S., Schnell, R.C., Higgins, R.W., Lowrimore, J., Kousky, V.E., Tinker, R., Thiaw, W., Chelliah, M., and Artusa, A. (2000) *Bull. Am. Meteorol. Soc.*, **81**.
9 Abdalla, A.A. and Verderk, K. (1968) *Neth. J. Agric. Sci.*, **16**, 71–76.
10 Weis, E. and Berry, J.A. (1988) *Symp. Soc. Exp. Biol.*, **42**, 329–346.
11 Stevens, M.A. and Rudich, J. (1978) *Hort. Sci.*, **13**, 673–678.
12 Sato, S., Peet, M.M., and Thomas, J.F. (2002) *J. Exp. Bot.*, **53**, 1187–1195.
13 Hazra, P., Samsul, H.A., Sikder, D., and Peter, K.V. (2007) *Int. J. Plant Breed.*, **1** (1)
14 CGIAR (2003) Applications of molecular biology and genomics to genetic enhancement of crop tolerance to abiotic stresses – a discussion document. Interim Science Council Secretariat, FAO.
15 McWilliam, J.R. (1986) *Aust. J. Plant Physiol.*, **13**, 1–13.
16 IPCC (2001) Climate change 2001: Impacts, adaptation and vulnerability.

Intergovermental Panel on Climate Change, New York, USA.
17 AVRDC (1990) *Vegetable Production Training Manual*, Asian Vegetable Research and Training Center., Shanhua, Taiwan, p. 447.
18 Umezawa, T., Fujita, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) *Curr. Opin. Biotechnol.*, **17**, 113–122.
19 Opena, R.T. and Lo, S.H. (1981) Breeding for heat tolerance in heading Chinese cabbage, in *Proceedings of the 1st International Symposium on Chinese Cabbage* (eds N.S. Talekar and T.D. Griggs), AVRDC, Shanhua, Taiwan.
20 Opena, R.T., Green, S.K., Talekar, N.S., and Chen, J.T. (1989) Genetic improvement of tomato adaptability to the tropics: progress and future prospects, in *Tomato and Pepper Production in the Tropics* (ed. S.K. Green), AVRDC, Shanhua, Taiwan, pp. 70–85.
21 Hanson, P.M., Chen, J.T., and Kuo, C.G. (2002) *Hort. Sci.*, **37**, 172–175.
22 Opena, R.T., Green, S.K., Talekar, N.S., and Chen, J.T. (1990) Genetic improvement of tomato adaptability to the tropics, in *Integrated Pest and Management Practices for Tomato and Pepper in the Tropics* (ed. S.K. Green), AVRDC, Shanhua, Taiwan, pp. 70–85.
23 Opena, R.T., Chen, J.T., Kuo, C.G., and Chen, H.M. (1992) Genetic and physiological aspects of tropical adaptation in tomato, in *Adaptation of Food Crops to Temperature and Water Stress* (ed. C.G. Kuo), AVRDC, Shanhua, Taiwan, pp. 321–334.
24 Metwally, E., El-Zawily, A., Hassan, N., and Zanata, O. (1996) Inheritance of fruit set and yields of tomato under high temperature conditions in Egypt. First Egyptian-Hungarian Horticultural Conference, Vol. I.
25 Villareal, R.L., Lai, S.H., and Wong, S.H. (1978) *Hort. Sci.*, **13**, 479–481.
26 Thomashow, M. (2001) *Plant Physiol.*, **125**, 89–93.
27 Steponkus, P., Uemura, M., Joseph, R.A., Gilmour, S.J., and Thomashow, M.F. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 14570–14575.
28 Zhang, J.Z., Creelman, R.A., and Zhu, J.K. (2004) *Plant Physiol.*, **135**, 615–621.
29 Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., and Samaha, R.R. (2000) *Science*, **290**, 2105–2110.
30 Bohnert, H.J., Ayoubi, P., Borchert, C., Bressan, R.A., Burnap, R.L., Cushman, J.C., Cushman, M.A., Deyholos, M., Fischer, R., and Galbraith, D.W. (2001) *Plant Physiol. Biochem.*, **39**, 295–311.
31 Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. (2001) *Plant Cell*, **13**, 61–72.
32 Chen, T.H.H. and Murata, N. (2002) *Curr. Opin. Plant Biol.*, **5**, 250–257.
33 Fowler, S. and Thomashow, M.F. (2002) *Plant Cell*, **14**, 1675–1690.
34 Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., and Harper, J.F. (2002) *Plant Physiol.*, **130**, 2129–2141.
35 Ingram, J. and Bartels, D. (1996) *Annu. Rev. Plant Biol.*, **47**, 377–403.
36 Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 1035–40.
37 Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M., and Thomashow, M.F. (1998) *Plant J.*, **16**, 433–442.
38 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) *Plant Cell*, **10**, 1391–1406.
39 Thomashow, M.F., Gilmour, S.J., Stockinger, E.J., Jaglo-Ottosen, K.R., and Zarka, D.G. (2001) *Physiol. Plant.*, **112**, 171–175.
40 Thomashow, M.F. (1998) *Plant Physiol.*, **118**, 1–7.
41 Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998) *Science*, **280**, 104–106.
42 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) *Nat. Biotech.*, **17**, 287–291.

43 Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D., and Thomashow, M.F. (2000) *Plant Physiol.*, **124**, 1854–1865.

44 Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T., and Thomashow, M.F. (2001) *Plant Physiol.*, **127**, 910–917.

45 Hsieh, T.H., Lee, J.T., Yang, P.T., Chiu, L.H., Charng, Y.Y., Wang, Y.C., and Chan, M.T. (2002) *Plant Physiol.*, **129**, 1086–1094.

46 Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F., and Zhang, J.Z. (2002) *Plant Physiol.*, **130**, 639–648.

47 Guiltinan, M.J., Marcotte, W.R., and Quatrano, R.S. (1990) *Science*, **250**, 267–271.

48 Mundy, J., Yamaguchi-Shinozaki, K., and Chua, N.H. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 406–410.

49 Hobo, T., Kowyama, Y., and Hattori, T. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 15348–15353.

50 Choi, H.I., Hong, J.H., Ha, J., Kang, J.Y., and Kim, S.Y. (2000) *J. Biol. Chem.*, **275**, 1723–1730.

51 Finkelstein, R.R. and Lynch, T.J. (2000) *Plant Cell*, **12**, 599–609.

52 Lopez-Molina, L. and Chua, N.H. (2000) *Plant Cell Physiol.*, **41**, 541–547.

53 Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 11632–11637.

54 Kang, J.Y., Choi, H.I., Im, M.Y., and Kim, S.Y. (2002) *Plant Cell*, **14**, 343–357.

55 Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003) *Plant Cell*, **15**, 63–78.

56 Feder, M.E. and Hofmann, G.E. (1999) *Annu. Rev. Physiol.*, **61**, 243–282.

57 Ahrman, E., Gustavsson, N., Hultschig, C., Boelens, W., and Emanielsson, C.S. (2007) *Extremophiles*, **11**, 659–666.

58 Prieto-Dapena, P., Catano, R., Almoguera, C., and Jordano, J. (2006) *Plant Physiol.*, **142**, 1102–1112.

59 Schöffl, F., Präandl, R., and Reindl, A. (1998) *Plant Physiol.*, **117**, 1135–1141.

60 Nover, L., Bharti, K., Döring, P., Mishra, S.K., Ganguli, A., and Scharf, K.D. (2001) *Cell Stress Chaperones*, **6**, 177–189.

61 Morimoto, R.I. (1998) *Genes Dev.*, **12**, 3788–3796.

62 Mishra, S.K., Tripp, J., Winkelhaus, S., Tschiersch, B., Theres, K., Nover, L., and Scharf, K.D. (2002) *Genes Dev.*, **16**, 1555–1567.

63 Lee, J.H., Hübel, A., and Schöffl, F. (1995) *Plant J.*, **8**, 603–612.

64 Prändl, R., Hinderhofer, K., Eggers-Schumacher, G., and Schöffl, F. (1998) *Mol. Gen. Genet.*, **258**, 269–278.

65 Yamanouchi, U., Yano, M., Lin, H., Ashikari, M., and Yamada, K. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 7530–7535.

66 Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Qifa, Z., and Xiong, L. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 12987–12992.

67 Liu, K., Lei, W., Xu, Y., Chen, N., Ma, Q., Li, F., and Chong, K. (2007) *Planta*, **226**, 1007–1016.

68 Ryder, E.J. (2002) The new salad crop revolution, in *Trends in New Crops and New Uses* (eds J. Janick and A. Whipkey), ASHS Press, Alexandria, VA, pp. 408–412.

69 Vanjildorj, E., Bae, T.W., Riu, K.Z., Kim, S.Y., and Lee, H.Y. (2005) *Plant Cell Tissue Organ Cult.*, **83**, 41–50.

70 Chaves, M.M. and Oliveira, M.M. (2004) *J. Exp. Bot.*, **55**, 2365–2384.

71 Ort, D.R. (2001) *Plant Physiol.*, **125**, 29–32.

72 Rick, C.M. (1973) Potential genetic resources in tomato species: clues from observation in native habitats, in *Genes, Enzymes and Populations* (ed. A.M. Srb), Plenum Press, New York, pp. 255–269.

73 Maldonado, C., Squeo, F.A., and Ibacache, E. (2003) *Rev. Chil. Hist. Nat.*, **76**, 129–137.

74 Sánchez Peña, P. (1999) Leaf water potentials in tomato (*L. esculentum* Mill.) L. chilense Dun. and their interspecific F1. MSc Thesis, New Mexico State University, Las Cruces, NM, USA.

75 O'Connell, M.A., Medina, A.L., Sanchez Pena, P., and Trevino, M.B. (2007) Molecular genetics of drought resistance response in tomato and related

species, in *Genetic Improvement of Solanaceous Crops*, vol. 2 (eds M.K. Razdan and A.K. Mattoo), Science Publishers, Enfield USA, pp. 261–283.
76 Flowers, T.J. (2004) *J. Exp. Bot.*, **55**, 307–319.
77 Foolad, M.R. (2004) *Plant Cell Tissue Organ Cult.*, **76**, 101–119.
78 Cuartero, J., Bolarin, M.C., Asins, M.J., and Moreno, V. (2006) *J. Exp. Bot.*, **57**, 1045–1058.
79 Knapp, S. (2002) *J. Exp. Bot.*, **7**, 2001–2022.
80 Govindarajan, V.S. and Sathyanarayana, M.N. (1991) *Crit. Rev. Food Sci. Nutr.*, **29** (6), 435–474.
81 Monsereenusorn, Y., Kongsamut, S., and Pezalla, P.D. (1982) *Crit. Rev. Toxicol.*, **10**, 321–339.
82 Yildirim, E. and Guvenc, I. (2006) *Turk. J. Agric. Forestry*, **30**, 347–353.
83 Hong, J.K., Choi, H.W., Hwang, I.S., Kim, D.S., Kim, N.H., Choi, D.S., Kim, Y.J., and Hwang, B.K. (2008) *Planta*, **227** (3), 539–558.
84 Kim, T.-H., Ok, S.H., Kim, D., Suh, S.-C., Byun, M.O., and Shin, J.S. (2009) *Plant Sci.*, **176** (5), 635–642.
85 Chung, E., Park, J.M., Oh, S.-K., Joung, Y.H., Lee, S., and Choi, D. (2004) *Planta*, **220**, 286–295.
86 Cho, S.K., Kim, J.E., Park, J.-A., Eom, T.J., and Kim, W.T. (2006) *FEBS Lett.*, **580**, 3136–3144.
87 An, S.H., Sohn, K.H., Choi, H.W., Hwang, I.S., Lee, S.C., and Hwang, B.K. (2008) *Planta*, **228**, 61–78.
88 Seong, E.S., Choi, D., Cho, H.S., Lim, C.K., Cho, H.J., and Wang, M.-H. (2007) *J. Biochem. Mol. Biol.*, **40** (6), 952–958.
89 Seong, E.S. and Wang, M.-H. (2007) BMB reports, 86–91.
90 Choi, H.W., Lee, D.H., and Hwang, B.K. (2009) *MPMI*, **22** (11), 1389–1400.
91 Chatzidimitriadou, K., Irini, N.-O., Panagiotis, M., Rafael, P.-T., and Athanasios, T. (2009) *Electron. J. Biotechnol.*, **12** (4), 1–9.
92 Gantait, S., Mandal, N., and Das, P.K. (2010) *Am. J. Plant Physiol.*, **5**, 325–337.
93 Lannoy, G.D. (2001) Vegetable crops in tropical Africa 395–511, in *Crop Production in Tropical Africa* (ed. R.H. Raemaeks), Directorate General for International Cooperation (DGIC), Belgium, p. 1540.
94 Straub, R.W. and Emmett, B. (1992) Pests of monocotyledon crops, in *Vegetable Crop Pests* (ed. R.G. McKinlay), Macmillan Press., U.K, pp. 213–262.
95 FAO (1991) Food and Agriculture Organization, Rome.
96 Grover, A., Aggarwal, P.K., Kapoor, A., Katiyar-Agarwal, S., Agarwal, M., and Chandramouli, A. (2003) *Curr. Sci.*, **84** (3), 355–367.
97 Wang, W.X., Vinocur, B., Shoseyov, O., and Altman, A. (2001) *Acta Hortic.*, **560**, 285–92.
98 Xiong, L., Schumaker, K.S., and Zhu, J.K. (2002) *Plant Cell*, **14**, S165–S183.
99 Hendry, G.A.F. and Wallace, R.K. (1993) *New Phytol.*, **123**, 3–14.
100 Bonnett, G.D., Sims, I.M., John, J.A.S., and Simpson, R.J. (1994) *New Phytol.*, **127**, 261–269.
101 Suzuki, M. and Pollock, C.J. (1986) *Can. J. Bot.*, **64**, 1884–1887.
102 Carpita, N.C., Kanabus, J., and Housley, T.L. (1989) *J. Plant Physiol.*, **134**, 162–168.
103 Shiomi, N. (1989) *J. Plant Physiol.*, **134**, 151–155.
104 Pollock, C.J. (1984) *New Phytol.*, **96**, 527–534.
105 Vijn, I. and Smeekens, S. (1999) *Plant Physiol.*, **120**, 351–359.
106 Vijn, I., van Dijken, A., Sprenger, N., van Dun, K., Weisbeek, P., Wiemken, A., and Smeekens, S. (1997) *Plant J.*, **11**, 387–398.
107 Sinclair, P.J., Blakeney, A.B., and Barlow, E.W.R. (1995) *J. Sci. Food Agric.*, **69**, 203–209.
108 Moshfegh, A.J., Friday, J.E., Goldman, J.P., and Chug Ahuj, J.K. (1999) *J. Nutr.*, **129**, 1407S–1411S.
109 Shiomi, N., Onodera, S., and Sakai, H. (1997) *New Phytol.*, **136**, 105–113.
110 Kahane, R., Vialle-Guerin, E., Boukema, I.I., Tzanoudakis, D., Bellamy, C., Chamaux, C., and Kik, C. (2001) *Environ. Exp. Bot.*, **45**, 73–83.
111 Pak, C., van der Plas, L.H.W., and de Boer, D. (1995) *Physiol. Plant.*, **94**, 277–283.

112 Wurr, D.C.E., Hand, D.W., Edmondson, R.N. *et al.* (1998) *J. Agric. Sci.*, **131**, 125–133.

113 Benkeblia, N., Varoquaux, P., Shiomi, N., and Sakai, H. (2002) *Int. J. Food Sci. Technol.*, **37**, 169–175.

114 Benkeblia, N., Varoquaux, P., Gouble, B., and Selselet-Attou, G. (2000) *J. Sci. Food Agric.*, **80**, 1772–1778.

115 Darbyshire, B. (1978) *J. Hortic. Sci.*, **53**, 195–201.

116 Hincha, D.K., Hellwege, E.M., Heyer, A.G., and Crowe, J.H. (2000) *Eur. J. Biochem.*, **267**, 535–540.

117 Eagles, C.F. (1967) *Ann. Bot.*, **31**, 645–651.

118 Pontis, H.G. (1989) *J. Plant Physiol.*, **134**, 148–150.

119 Tang, W. and Newton, R.J. (2005) *Plant Cell Rep.*, **24**, 581.

120 Gemici, M., Unal, D., Azeri, F.N., and Tan, K. (2006) *J. Food Sci.*, **29**, 13.

121 Chattopadhyay, M.K., Tabor, C.W., and Tabor, H. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 10330.

122 Theiss, C., Bohley, P., and Voigt, J. (2002) *Plant Physiol.*, **128**, 1470.

123 Zeevaart, J.A.D. and Creelman, R.A. (1988) *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **39**, 439.

124 Robertson, J.M., Yeung, E.C., Reid, D.M., and Hubick, K.T. (1990) *J. Exp. Bot.*, **41** (224), 339.

125 Amaral Da Silva, E.A., Toorop, P.E., Van Lammeren, A.A.M., and Hilhorst, H.W.M. (2008) *Ann. Bot.*, **102**, 425.

126 Mahajan, A. and Sharma, S. (2009) *Indian J. Exp. Biol.*, **47**, 136–139.

127 Levitt, J. (1980) *Responses of Plants to Environmental Stresses, II. Water, Radiation, Salt and Other Stresses*, Academic Press, New York.

128 Shanon, M.C. (1979) *Hort. Sci.*, **5**, 587–589.

129 Gupta, I.C. and Yadav, J.S.P. (1986) *J. Indian Soc. Soil Sci.*, **3**, 379–386.

130 Malach, Y.D., Pasternak, D., Mendlinger, S., Borovic, I., Salam, N.E., and Malach, D. (1989) *Agric. Water Manage.*, **6**, 201–215.

131 Al-Islali, N.Y. and Al-Bahrany, A.M. (1994) *Alex. J. Agri. Res.*, **39** (3), 597–610.

132 Yokoi, S., Bressan, R.A., and Hasegawa, P.M. (2002) JIRCAS Working Report 25–33.

133 Cramer, R.C., Luchli, A., and Polito, V.S. (1985) *Plant Physiol.*, **79**, 207–211.

134 Hasegawa, P.M., Bressan, R.A., Zhu, J.K., and Bohnert, H.J. (2000) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **51**, 463–499.

135 Zhu, J.K. (2001) *Trends Plant Sci.*, **6**, 66–71.

136 Noctor, G. and Foyer, C.H. (1998) *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **49**, 249–279.

137 Khan, M.H. and Panda, S.K. (2002) *Biol. Plant.*, **45**, 525–627.

138 Halliwell, B. and Gutteridge, J.M.C. (1986) *Arch. Biochem. Biophys.*, **246**, 501–514.

139 Mittler, R. (2002) *Trends Plant Sci.*, **7**, 405–410.

140 Blokhina, O., Virolainen, E., and Fagerstedt, K.V. (2003) *Ann. Bot.*, **91**, 179–194.

141 Roxas, V.P., Smith, R.K., Allen, E.R., and Allen, R.D. (1997) *Nat. Biotechnol.*, **15**, 988–991.

142 Millar, A.H., Mittova, V., and Kiddle, G. (2003) *Plant Physiol.*, **133**, 443–447.

143 Hussain, T.M., Hazara, M., Sultan, Z., Saleh, B.K., and Gopal, G.R. (2008) *Biotechnol. Mol. Biol. Rev.*, **3**, 8–13.

144 Marrs, K.A. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 127–158.

145 Galle, Á., Csiszár, J., Secenji, M., Tari, I., Guóth, A., Györgyey, J., and Erdei, L. (2008) *Acta Biol. Szegediensis*, **52**, 95–96.

146 Boot, K.J.M., van der Zaal, B.J., Velterop, J., Quint, A., Mennes, A.M., Hooykaas, P.J.J., and Libbenga, K.R. (1993) *Plant Physiol.*, **102**, 513–520.

147 Sappl, P.G., Onate-Sanchez, L., Singh, K.B., and Millar, A.H. (2004) *Plant Mol. Biol.*, **54**, 205–219.

148 Marrs, K.A. and Walbot, V. (1997) *Plant Physiol.*, **113**, 93–102.

149 Hossain, M.D., Rohman, M.M., and Fujita, M. (2007) *J. Crop Sci. Biotechnol.*, **10**, 21–28.

150 Rohman, M.M., Hossain, M.D., Suzuki, T., Takada, G., and Fujita, M. (2009) *Acta Physiol. Plant.*, **31**, 301–309.

151 Rohman, M.M., Suzuki, T., and Fujita, M. (2009) *Aust. J. Crop Sci.*, **3**, 28–36.

152 Rohman, M.M., Uddin, M.S., and Fujita, M. (2010) *POJ*, **3** (1), 28–34.

153 Mueller, L.A., Godman, C.D., Silady, R.A., and Walbot, V. (2000) *Plant Physiol.*, **123**, 1561–1570.

154 Cummins, I., O'Hagen, D., Jablonkai, I., Cole, D.J., Hehn, A., Werck-reichhart, D., and Edwards, R. (2003) *Plant Mol. Biol.*, **52**, 591–603.

155 Salama, K.H.A. and Mutawa, M.M.A. (2009) *Int. J. Agric. Biol.*, **11**, 639–642.

156 Cisneros-Zevallos, L. (2003) *J. Food Sci.*, **68**, 1560–1565.

157 Chang, P.T. and Randle, W.M. (2005) *J. Plant Nutr.*, **28**, 1755–1766.

158 Barandiaran, X., Martin, N., Alba, C., Rodriguez-Conde, M., Di Pietro, A., and Martin, J. (1999) *In Vitro Cell. Dev. Plant*, **35**, 466–469.

159 Barandiaran, X., Martin, N., Rodriguez-Conde, M., Di Pietro, A., and Martin, J. (1999) *Hort. Sci.*, **34**, 348–349.

160 Barandiaran, X., Martin, N., Rodriguez-Conde, M., Di Pietro, A., and Martin, J. (1999) *Plant Cell Rep.*, **18**, 434–437.

161 Martin-Urdiroz, N., Garrido-Gala, J., Martin, J., and Barandiaran, X. (2004) *Plant Cell Rep.*, **22**, 721–724.

162 Eady, C., Weld, R., and Lister, C. (2000) *Plant Cell. Rep.*, **19**, 376–381.

163 Eady, C., Davis, S., Catanach, A., Kenel, F., and Hunger, S. (2005) *Plant Cell. Rep.*, **24**, 209–215.

164 Zheng, S.J., Henken, B., Ahn, Y., Krens, F., and Kik, C. (2004) *Mol. Breed.*, **14**, 293–307.

165 Vijn, I., van Dijken, A., Luscher, M., Bos, A., Smeets, E., Weisbeek, P., Wiemken, A., and Smeekens, S. (1998) *Plant Physiol.*, **117** (4), 1507–1513.

38
Vegetable Crops: Improvement of Tolerance to Adverse Chemical Soil Conditions by Grafting

Giuseppe Colla, Youssef Rouphael, and Mariateresa Cardarelli

Owing to limited availability of arable land and the high market demand for vegetables around the world, Solanaceae and Cucurbitaceae crops are frequently cultivated under unfavorable soil conditions. These include salinity, alkalinity, heavy metals, and excessive amount of trace elements. Plants exposed to adverse chemical soil conditions exhibit various physiological and biochemical disorders leading to stunted growth and severe yield loss. One way to avoid or reduce losses in production caused by adverse soil chemical conditions in vegetables would be to graft them onto rootstocks capable of reducing the effect of external stresses on the shoot. Grafting is an integrative reciprocal process and, therefore, both scion and rootstock can influence tolerance of grafted plants to adverse soil chemical conditions. Grafted plants grown under adverse soil chemical conditions often exhibited greater growth and yield, higher photosynthesis, better nutritional status, and lower accumulation of Na^+ and/or Cl^-, heavy metals, and excessive amount of trace elements in shoots than ungrafted or self-grafted plants. This chapter gives an overview of the recent literature on the response of grafted plants to adverse soil chemical conditions and the mechanisms of tolerance to adverse soil chemical conditions in grafted plants related to the morphological root characteristics and the physiological and biochemical processes. The chapter will conclude by identifying several prospects for future research aiming to improve the role of grafting in vegetable crops grown under abiotic stress conditions.

38.1
Introduction

Soil chemical factors such as salinity, alkalinity, heavy metals, and excessive amount of trace elements are common abiotic stresses limiting crop productivity in many parts of the world. At present, a third of irrigated land in the world is affected by salinity and alkalinity problems [1, 2], while many soils are contaminated by heavy metals.

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According to the US Salinity Laboratory, when the electrical conductivity (EC) of solution extracted from a soil at its saturation water content is greater than 4 dS m^{-1} and the exchangeable sodium percentage (ESP) is less than 15, the soil is considered saline. Salinity occurs in both nonirrigated and irrigated lands as a result of evapotranspiration of saline underground water or due to the use of irrigation water of poor quality. Soil salinization is common especially in arid and semiarid regions where the amount of rainfall is insufficient for leaching. The salinization process is particularly evident under greenhouse conditions where the lack of leaching by rainfall and the high fertilizer application rates result in a dramatic increase in the electrical conductivity value of soils, especially when poor-quality water is used [3]. Although NaCl is usually the most abundant salt in saline soil, other elements (e.g., Ca^{2+}, Mg^{2+}, K^{+}, SO_4^{2-}, and NO_3^{-}) can be presented in different combinations depending on the source of salinity and the solubility of the salts [4]. Moreover, the saline water may contain high concentrations of trace elements that can be harmful to most vegetable crops (e.g., B > 1–2 mg L^{-1}). Under saline conditions, crop performance may be adversely affected by water deficit arising from the low water potential of the soil solution (osmotic effect) and by salinity-induced nutritional disorders associated with excessive ion uptake or nutrient imbalance by nutrient availability, competitive uptake, and transport or partitioning within the plant (ionic effect) [5, 6]. The pH of saline soil is usually around neutrality or slightly alkaline. However, higher pH values (>8) are observed in saline soils with ESP greater than 15 (saline–sodic soils) due to the high content of sodium carbonate. Plant growth in sodic soils is depressed mainly by high pH and bicarbonate, and often by low soil permeability to water, poor aeration, and mechanical impedance. However, alkaline soils are often not associated with salinity, especially when the source of alkalinity is $CaCO_3$ that buffers the soil in the pH range 7.5–8.5 (calcareous soils). Calcareous soils are generally characterized by low bioavailability of plant nutrients, high concentrations of $CaCO_3$ and soil solution HCO_3^{-}, high pH, and almost no exchangeable H^{+} [7, 8]. Bicarbonate ions reduce the plant growth by interfering negatively with the uptake of macroelements, in particular P, K, and Mg [9]. For instance, in alkaline soils, P is largely unavailable to plants due to the formation of metal complexes (e.g., Ca–P and Mg–P), rendering P only sparingly soluble. Moreover, the concentration of HCO_3^{-} interacts strongly with the availability of several micronutrients, especially Fe^{2+}, and it is often considered to be the primary factor responsible for chlorosis of plants on calcareous soils [10] leading to serious yield and quality losses. Reduction in iron availability is due to the incapacity of sensitive plants to acquire and to transport iron toward shoots. Iron deficiency reflects upon the physiology and biochemistry of the whole plant, as iron is an important cofactor of many enzymes, including those involved in the biosynthetic pathway of chlorophylls [7]. Thus, under iron deficiency conditions, the reduction in leaf iron concentration is often accompanied by a marked reduction of chlorophyll levels [11, 12], by a significant, although less intense, decrease in the chlorophyll fluorescence [11, 12] and by a reduction in photosynthesis [7].

Contamination of soil and water by heavy metals and excessive amount of trace elements is one of the most troublesome environmental problems faced by mankind

nowadays. Heavy metals are getting importance for their nondegradable nature and often accumulate through tropic level causing a deleterious biological effect. Anthropogenic activities such as mining, ultimate disposal of treated and untreated waste effluents containing toxic metals as well as metal chelates [13] from different industries, and the indiscriminate use of heavy metal-containing fertilizers (e.g., triple superphosphate, animal wastes, and sewage sludge) and pesticides (e.g., Cu-containing fungicides) in agriculture resulted in contamination of soils and in deterioration of water quality, rendering serious environmental problems posing threat to human beings [14]. Some of the metals such as Cu, Fe, Mn, Ni, and Zn are essential as micronutrients for plants, while many other metals such as Cd, Cr, and Pb have no known physiological activity, but they are proved detrimental to plant growth beyond a certain limit, which is very much narrow for some elements such as Cd (0.01 mg/L), Pb (0.10 mg/L), and Cu (0.050 mg/L) [15]. Inhibition of root elongation is in many cases the most sensitive parameter of heavy metal toxicity. Excessive levels of heavy metals in plant tissues can also cause a range of morphological and physiological disorders, such as reduction in shoot growth [16], photosynthetic activity [17], and uptake of mineral nutrients [18]. Moreover, it may result in chlorosis and necrosis, and damage to plasma membrane permeability that leads to ion leakage [19]. Finally, vegetables cultivated in contaminated soils may exhibit high levels of heavy metals in the edible parts posing serious health risks to humans [20]. Numerous attempts have been made to overcome the problems due to adverse soil chemical conditions by traditional breeding programs, but commercial success has been very limited. At present, the major efforts are being directed toward the genetic transformation of plants. Although the expression of a single gene seems to lead in some cases to an improvement in the crop adaptation to some adverse soil chemical factors [21], the development of tolerant genotypes normally requires the transfer of several genes due to the multigenic trait of abiotic stress tolerance [22]. As a rapid alternative to the relatively slow breeding methodology aimed at increasing vegetable crop tolerance to an abiotic stress, grafting of high-yield genotypes onto selected rootstocks could be a promising tool. Grafting is commonly applied to vegetable crops belong to Solanaceous crops (tomato, eggplant, and pepper) and Cucurbits (watermelon, melon, and cucumber) in Japan, Korea, China, and several European and American countries [23]. The main purpose of grafting is to control soil-borne diseases and nematodes [24]; in addition, grafting may increase the nutrient and water use efficiency (WUE), enhance plant vigor and yield, and improve tolerance to environmental stresses such as high salinity, low and high temperatures, drought, flooding-induced hypoxia, alkalinity, and excessive amount of heavy metals and trace elements [23].

In this chapter, we emphasize the potentiality of vegetable grafting as a tool to mitigate the detrimental effects of adverse soil chemical conditions such as salinity, alkalinity, heavy metals, and excessive amount of trace elements on vegetable crop performances. The role of grafting in the improvement of growth, yield, and product quality under such soil abiotic stresses is reported. Various mechanisms involved in the increased tolerance to salinity, alkalinity, heavy metals, and excessive amount of trace elements are also discussed.

38.2 Salinity

38.2.1 Effects on Grafted Plants

38.2.1.1 Growth and Yield

It is well established that crop growth and yield decrease with increasing salinity [25]. Reduced yield under saline treatments is attributed to a rapid, osmotic phase that inhibits growth of young leaves, and a slower, ionic phase that accelerates senescence of mature leaves [6]. Osmotic stress can also induce premature senescence via stomatal closure and carbohydrate accumulation in source tissues due to decreased demand from sink organs. Improvement of growth and yield was observed in many grafting combinations of fruit vegetables grown under saline conditions. Moreover, it has been demonstrated that the level of tolerance of grafting combinations depends on the salt type and concentration, exposure time, and growing conditions. Generally, the positive effect induced by rootstock on shoot saline tolerance increases with the level of stress as observed in tomato [26], where grafting "Moneymaker" onto either "Radja" or "Pera" improved tomato fruit yield compared to self-grafted plants of "Moneymaker" when plants were grown at 50 mM NaCl, whereas there was no effect of either rootstocks or grafting *per se* on fruit yield in the absence of or at 25 mM NaCl. The yield increase over self-grafted plants was around 40% whereas in the earlier study [27] using a different scion ("Jaguar"), the increase was 80% at the same salt concentration indicating a different salt tolerance of the genotypes. These results suggest that the salt tolerance of the shoot depends on the root system, independent of the genotype used as a scion, although the positive effect of rootstock may show a different degree depending on the higher or lower exclusion ability of the shoot genotype. Similarly, in eggplant (*Solanum melongena* L.) grafting cultivar "Suqiqie" onto "Torvum Vigor" (*S. torvum* Swartz) improved the growth performance under saline stress conditions [28, 29]. The better crop performance in grafted Solanaceous crops grown under saline conditions has also been recorded on several Cucurbits such as watermelon, melon, and cucumber. Grafting watermelon "Fantasy" cultivar onto plants "Strongtosa" rootstock (*Cucurbita maxima* Duch. × *C. moschata* Duch.) reduced the decrease in shoot weight and leaf area caused by the increase in salinity in comparison with ungrafted plants [30]. Moreover, other experiments demonstrated that grafted "Crimson Tide" watermelon onto *C. maxima* and two *Lagenaria siceraria* rootstocks had higher plant growth than ungrafted plants under saline conditions (8.0 dS m^{-1}, [31]). In cucumber (*Cucumis sativus* L.), grafting cultivar "Jinchun No. 2" onto bottle gourd rootstock "Chaofeng 8848" (*L. siceraria* Standl.) alleviated the negative effect of salinity on shoot dry weight [32]. In a similar study, cucumber plants cultivar "Jinchun No. 2" grafted onto figleaf gourd (*C. ficifolia* Bouché) and "Chaofeng Kangshengwang" had higher fruit number and marketable fruit yield compared to the self-grafted plants at all salt levels (30 and 60 mM NaCl). Similarly, two melon cultivars (*C. melo* L.) grafted onto three hybrids of squash (*C. maxima* Duch. × *C. moschata* Duch.) exhibited higher yield compared to ungrafted ones when grown

under saline conditions (4.6 dS m^{-1} [33]). However, other researchers [34, 35] recorded that the sensitivity to salinity was similar between grafted and ungrafted melon plants as a result of the different *Cucurbita* rootstocks used in these studies. Salt tolerance of grafted plants can vary significantly in relation to the salt composition and growing system. For instance, cucumber plant "Jinchun No. 2" grafted onto "Chaofeng Kangshengwang" (*L. siceraria* Standl.) was tolerant to salinity when grown in hydroponics using NaCl as salt source, while "Chaofeng Kangshengwang" failed to increase grafted plant tolerance to salinity when grown in substrate culture using macronutrients as salinity source [36].

38.2.1.2 **Photosynthesis and Water Relations**

Under saline conditions, the low osmotic potential of soil solution restricts water availability and water uptake and thus reduces the root hydraulic conductance and causes a significant increase in the stomatal resistance and reduction in CO_2 photosynthetic assimilation. Stomatal opening and photosynthesis, which are K^+-dependent physiological processes, can also be reduced by NaCl saline conditions as a result of the decreased absorption of some nutrients (e.g., K) and the increased content of the Na^+ and Cl^- in the leaves. Under saline conditions, salt-tolerant grafted plants exhibit higher photosynthetic rate per unit area and higher leaf area resulting in a greater photosynthetic capacity of the plant than ungrafted or self-grafted plants. For instance, in a watermelon experiment [37], the leaf area and the net assimilation of CO_2 under saline conditions were higher in grafted plants of cultivar Tex onto *Cucurbita* hybrid "Ercole" than in ungrafted "Tex" plants. Similarly, it was demonstrated [38] that grafted cucumber plants had higher net photosynthesis, stomatal conductance, and intercellular CO_2 concentrations under NaCl stress than self-rooted plants. Moreover, under moderate and severe salt stresses, tomato-grafted plants of "Hezu903" onto "Zhezhen" rootstock showed higher net CO_2 assimilation rate than nongrafted and self-grafted plants [39]. Water use efficiency, calculated as the ratio of net assimilation of CO_2 to transpiration, usually increased in moderately salt-stressed plants, owing to the fast decrease in transpiration rate. For instance, grafting tomato "Hezu903" onto "Zhezhen" rootstock increased the WUE under saline conditions in comparison to the ungrafted and self-grafted plants [39]. The higher WUE is important for salt tolerance since a high WUE may reduce the uptake of salt and alleviate the water deficiency induced by salinity [40, 41]. Water content maintenance and transpiration are crucial to plants under salinity stress. Water deficit associated with salinity can increase the leaf water content as a result of the transpiration rate reduction due to the stomatal closure. For instance, at 100 mM of NaCl, higher leaf water content was observed in grafted tomato plants of "UC-82B" onto "Kyndia" rootstock compared to the self-grafted plants; the better leaf water content was associated with a lower shoot growth reduction in grafted plants [42].

38.2.1.3 **Fruit Quality**

In general, salinity reduces the yield of vegetable crops but in many instances improves their quality [43]. Many investigations have shown that increased salinity produces fruit with a higher content of sugars and organic acids, and higher dry

matter, providing a basis for better taste and high nutritional value. Grafting can increase or decrease the fruit quality depending on the scion–rootstock combination, the salt composition, and the growing conditions [44]. For instance, the dry matter, soluble solid content, and titratable acidity were lower in melon fruits (*C. melo* L.) of cultivar "Cyrano" grafted onto "P360" *Cucurbita* hybrid rootstock than in ungrafted ones regardless of the level of salinity [35]. In other experiments on tomato, the soluble solid content of fruits was similar in both grafted and ungrafted tomato plants and increased with NaCl stress level. On the contrary, it has been reported that grafting tomato cultivar "Moneymaker" onto "Radia" rootstock increased both yield and fruit quality parameters (soluble solids and titratable acidity) in comparison to self-grafted "Moneymaker" grown under saline conditions. Similarly, grafting cucumber cultivar "JinchunNo. 2" onto "Figleaf Gourd" (*C. ficifolia* Bouché) and "Chaofeng Kangshengwang" (*L. siceraria* Standl.) improved fruit quality under NaCl stress owing to an increase in contents of soluble sugar and titratable acidity and a decrease in the percentage of nonmarketable fruits [32]. It is interesting to note that in the cucumber experiment, the detrimental effects of *Cucurbita* rootstocks on fruit quality observed in the previous melon experiment was compensated by an increase in soluble sugar under salt stress, which was probably a consequence of a lower accumulation of saline ions (Na^+ and Cl^-) that led to a high accumulation of soluble sugar involved in the osmotic adjustment.

Since saline stress activates a physiological antioxidative response [45], it has been reported that ascorbic acid levels increase with salinity, as part of the detoxification of free radicals, and similarly moderate salt stress enhance the level of other antioxidants such as carotenoids (e.g., lycopene and β-carotene), which have been recognized as beneficial in preventing widespread human diseases, including cancer. Grafting can enhance the content of antioxidants in fruits depending on grafting combinations and salt concentration as observed in tomato, where the concentration of ascorbic acid in fruit juice of cultivar "Fanny" remained unchanged with grafting at 0 mM NaCl [46], whereas when NaCl was increased to 30 mM a significant increase was observed for grafted "Fanny" plants onto tomato rootstock "AR-9704." Moreover, an increasing in carotenoids (lycopene and β-carotene) was also observed in two tomato cultivars ("Fanny" and "Goldmar") grafted onto a tomato hybrid rootstock "AR-9704" under saline conditions. Similarly, vitamin C increased by grafting cucumber cultivar "JinchunNo." onto "Figleaf Gourd" and "Chaofeng Kangshengwang" in comparison to self-grafted plants, whether saline-challenged or not. Grafting can improve the mineral content of the fruits under saline conditions, which is interesting from a nutritional point of view because fruits and vegetables are important source of minerals in the human diet (e.g., 35, 24, and 11%, respectively, of the total K, Mg, and P dietary intake of humans) [47]. For instance, it has been reported that under saline conditions fruit K content was higher in cucumber cv. "Jinchun No." grafted onto "Figleaf Gourd" and "Chaofeng Kangshengwang" in comparison to self-grafted plants. Moreover, it has also been observed that salt-tolerant grafting combinations exhibited a decrease in Na and/or Cl contents in fruits in comparison to the self-grafted plants, which represents a positive quality aspect due to the negative effects of high dietary intake of Na and Cl on human health.

38.2.2 Mechanisms of Salt Tolerance in Grafted Plants

38.2.2.1 Root Characteristics

Generally, salinity induces a rapid reduction in root growth and an increase in root to shoot dry weight ratio due to a greater reduction in shoot growth; the high root to shoot ratio under saline conditions appears to be an adaptive strategy to increase the nutrient uptake and the ratio of water absorption by water-transpiring organs. Maintaining root growth under saline conditions was correlated with salt tolerance in tomato. Salt-tolerant grafted plants often exhibit a better root growth and higher root to shoot ratio than ungrafted or self-grafted plants. For example, grafted tomato plants had a less decrease in root dry mass at 100 and 150 mM of NaCl than ungrafted plants [39]. Similar results were observed in grafted cucumber plants [32, 48] and in grafted watermelon plants [31, 37] exposed to salt stress. Therefore, the better growth performance of grafted vegetable crops could be explained, at least to some extent, in terms of root growth under salinity stress.

38.2.2.2 Salt Exclusion and Root Retention

The main long-term damage caused by salinity in glycophyte crops is the excessive accumulation of Na^+ and Cl^- in leaves that causes a wide variety of physiological and biochemical alterations inhibiting plant growth and production. The increased salt tolerance of grafted vegetables has often been associated with lower Na^+ and/or Cl^- contents in the shoot. Two mechanisms could explain the decrease in shoot toxic ion (Na^+ and/or Cl^-) concentrations in grafted plants: toxic ion exclusion by the roots, and toxic ion retention and accumulation within the rootstock. Root retention involves the storage of Na^+ and/or Cl^- in vacuoles, which can protect cytosolic enzymes from the damaging effects of salt accumulation [49]. The electrochemical H^+ gradients generated by H^+ pumps in the tonoplast (H^+-ATPase, H^+-PPase) provide the energy used by tonoplast-bound Na^+/H^+ antiporters (NHX) to couple the passive movement of H^+ to the active movement of Na^+ into the vacuole [50]. The H^+-ATPase and H^+-PPase activities of root tonoplast membrane are less inhibited under NaCl stress in salt-tolerant grafted plants than in self-grafted plants as observed in tomato experiment [51]. Experiments conducted on melon plant grafted onto different hybrids of squash (*C. maxima* Duch. × *C. moschata* Duch.) revealed that the concentrations averaged 11.7 times those in the shoots of plants grafted with pumpkin rootstocks. Quantitative analysis indicated that Na exclusion of squash hybrid roots plays a more significant role in its restricted accumulation in the shoot of grafted plants, compared to its retention in the roots: Na exclusion was 69–79%, while the Na root retention was only 37–54% [52].

38.2.2.3 Osmotic Adjustment

Salt tolerance and further growth in a saline soil require a reduction in internal plant water potential below that of the soil in order to maintain turgor and water uptake. In fruit vegetables in which salt exclusion (glycophytes) is the principal mechanism of salt tolerance, either the synthesis of metabolically compatible solutes (e.g., sucrose,

proline, and glycine betaine) or the uptake of inorganic ions (e.g., K^+, Ca^{2+}, and NO_3^-) must be increased (osmotic adjustment). Unlike Na^+ and Cl^-, these osmotically active solutes are not harmful to enzymes and other cellular structures even at high concentrations (hence "compatible solutes"). Although the biosynthesis of organic compatible solutes is energetically more expensive than the accumulation of Na^+ and Cl^- take-up from the soil, plants can benefit from the reduction of the detrimental effects induced by high accumulation of Na^+ and Cl^-. Moreover, compatible osmolytes may protect plants by scavenging oxygen-free radicals caused by salt stress [53, 54]. It has been reported that salt-tolerant grafted plants of tomato and cucumber exhibited a better osmotic adjustment under NaCl stress through a higher accumulation of soluble sugars and proline in leaves than self-grafted plants [32]. Moreover, grafted plants have higher leaf K^+ accumulation, which seems related to the higher salt tolerance than self-grafted plants [32, 48].

38.2.2.4 Antioxidant Defense System

Salt stress reduces the photosynthesis rate increasing the formation of reactive oxygen speciess (ROS) such as superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2). These ROS are highly reactive and can seriously disrupt normal metabolism through oxidative damage to lipids, proteins, and nucleic acids [55]. Plants have evolved an efficient defense system by which the ROS is scavenged by enzymatic and nonenzymatic antioxidant defense mechanisms.

Enzymatic antioxidants include superoxide dismutase, catalase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase (GR). The most commonly known nonenzymatic antioxidants are glutathione (GSH), ascorbate (AsA), carotenoids, and tocopherols [55, 56]. An efficient antioxidant system is an important factor for the enhanced salt tolerance of grafted plants. For instance, the increased salt tolerance of cucumber plants grafted onto *C. ficifolia* was associated with the increased superoxide dismutase and peroxidase activities under saline conditions induced by major nutrients [36]. Similarly, the higher antioxidant capacity of grafted plants under salt stress has been observed in other fruit-bearing vegetables such as tomato, eggplant, and watermelon [39, 48]. Nonenzymatic antioxidants were also found to contribute to the salinity tolerance in grafted vegetables. The glutathione and ascorbate contents in the leaves of grafted eggplants are found to be significantly higher than those in self-grafted plants under NaCl stress [28].

38.2.2.5 Phytohormone Biosynthesis

Changes in phytohormones or their precursors' concentrations, such as cytokinins (CKs), abscisic acid (ABA), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and auxin indole 3-acetic acid (IAA), are associated with a response of plants to salinity. It has been suggested that at least part of the growth depression by salinity was caused by inadequate phytohormone production. Generally, the levels of CKs decrease while ABA increases in response to salinity. Abscisic acid plays a central role both in root to shoot and cellular signaling under salt stress and in the regulation of stomatal conductance. Under salt stress, a transient loss of leaf turgor stimulates ABA

synthesis and causes stomatal closures stimulating leaf senescence. CKs are assumed to be synthesized mainly in the roots and transported to the shoots via the xylem. CKs are implicated in controlling both shoot growth and leaf senescence. Some rootstocks exhibited a higher CKs biosynthesis that improved salt tolerance of scion by increasing vegetative and fruit growth and by delaying leaf senescence and maintaining stomatal conductance and PSII efficiency, thereby avoiding or delaying the accumulation of toxic ions [57]. Moreover, the ratio between CKs and ACC was positively correlated with leaf growth and PSII efficiency in a grafting tomato experiments, where tomato cultivar was grafted onto rootstocks from a population of recombinant lines derived from *S. lycopersicum* × *S. cheemaniae* cross and grown under moderate saline conditions (75 mM NaCl) [58]. Phytohormones also play an important role in maintaining root growth under salt stress condition and in increasing root to shoot ratio. The greater partitioning of assimilates to roots under saline condition has been attributed to a decrease in CK concentrations and an induced basipetal transport of auxin from shoot to root with a concomitant change in the activity of the sink-related enzyme cell wall invertase. Moreover, the increase in root to shoot ratio due to a differential growth response of root (maintenance) and shoot (inhibition) under salinity was associated with a relative increase and decrease in the auxin IAA concentration, respectively [58]. Polyamines [putrescine (Put), spermidine (Spd), and spermine (Spm)] are small cationic molecules that accumulate in plants under salinity stress. They are involved in the regulation of many basic cellular processes such as DNA replication and transcription, cell proliferation, modulation of enzyme activities, membrane rigidity, and stabilization [59]. In grafted tomatoes and eggplants, it has been reported that the ABA and total polyamine contents are significantly higher than those of self-grafted plants under NaCl stress [60, 61]. In addition, the Spd and Spm contents, as well as (Spd + Spm)/Put value, were higher in grafted melon and cucumber plants than in self-grafted plants under NaCl stress [62].

38.3 Alkalinity

38.3.1 Effects on Grafted Plants

38.3.1.1 Growth and Photosynthesis

Researchers have demonstrated that plants respond to elevated $NaHCO_3$ concentrations in soil or in growing medium solution with decreased shoot and root growth [63–65]. Shoot growth inhibition is associated with a decrease in the number of leaves, fresh and dry mass, and shoot elongation [66]. For instance, significant depression in shoot and root biomass production was observed in bicarbonate-treated watermelon plants, and that effect varied as a function of grafting combination [67]. Under alkaline conditions (pH 8.1), shoot and root biomass weight reductions in comparison to control (pH 6.0) were significantly lower in watermelon cultivar Ingrid grafted onto *Cucurbita* rootstocks "PS1313" and "P360" than that in

ungrafted watermelon plants, whereas the root to shoot ratio of biomass increased in ungrafted plants. The former study showed that the watermelon plants grafted onto *Cucurbita* rootstocks had less change in root to shoot ratio than those grafted onto bottle gourd rootstocks and the ungrafted plants under alkaline conditions. The lower shoot reductions in grafted plants, especially in those grafted onto pumpkin rootstocks, was related to the capacity of maintaining a higher net CO_2 assimilation in response to bicarbonate stress compared to ungrafted plants. In addition to reduced net photosynthetic rates, leaf area decreased in response to an increase in alkalinity in the nutrient solution especially in ungrafted watermelon plants. The restriction of leaf area may be the result of the suppressed net photosynthetic rates since the latter effect reduces the available assimilates for leaf growth.

38.3.1.2 **Nutrient Uptake**

Alkaline soils represent a serious concern for iron acquisition by plants since under these conditions the range of inorganic iron availability is around 0.1–10% of the normal requirement for optimal plant growth [68]. Colla *et al.* [67] observed that roots of grafted and ungrafted watermelon plants accumulated larger amounts of Fe than leaves, suggesting that the critical process leading to chlorosis in alkaline soils is Fe uptake from the root apoplast into the symplast, which can be impaired by the alkaline apoplastic pH due to high bicarbonate concentration [10, 69]. Grafted watermelon onto pumpkin rootstocks enhanced the uptake and translocation of Fe toward the shoot in comparison with ungrafted plants. The higher uptake and accumulation of Fe in watermelon plants grafted onto pumpkins was the main mechanism that reduced the detrimental effect of alkalinity (Fe deficiency) on plant growth. Moreover, bicarbonate ions may interfere with the uptake and transport of other essential nutrients (e.g., P, K, and Mg) and thereby disturbing nutrient composition of plants [7]. On the basis of the nutrient composition of plant tissues, it has been demonstrated that watermelon grafted and ungrafted plants responded differently to pH level, as has been observed for growth parameters. For ungrafted plants, the high pH level (8.1) in the nutrient solution caused significant decrease in macronutrient leaf concentration especially for P compared to plants grafted onto pumpkin rootstocks. Consequently, the improved crop performance of watermelon plants grafted onto pumpkin rootstocks was attributed not only to their strong capacity to accumulate Fe in the aerial part under alkaline conditions but also to their ability to improve the uptake and transport of P to the shoot [67].

38.3.2
Mechanisms of Alkalinity Tolerance in Grafted Plants

38.3.2.1 **Root Exudation**

Plants respond to deficiency of many macro- and micronutrients with increased root exudation, for example, to, P [70], K [71], Zn [72], and Cu [73]. Since organic acids efficiently solubilize/mobilize many metal cations such as Ca, K, and Mg [74], Al and Fe [74, 75], and Mn [76], the purpose of this increased exudation could be to increase the solubilization of deficient nutrients. Certain rootstocks can improve the uptake of

several macro- and micronutrients under alkaline conditions through a greater exudation of organic acids. For instance, watermelon plants grafted onto pumpkin rootstocks exuded more citric and malic acids than ungrafted ones especially under bicarbonate-enriched solutions [67]. These results support the hypothesis that uptake of nutrients (e.g., P and Fe) from the nutrient solution by pumpkin rootstocks was facilitated by exudation of organic acids from roots. Similar results were observed in other experiments on *Cucurbita* plants where the root exudation of organic acids (especially citric acid) increased under P depletion leading to an enhanced P uptake especially in *C. pepo* ssp. *ovifera* [70].

38.3.2.2 **Root-Reducing Capacity**

Higher plants have developed various specific and nonspecific mechanisms to increase the solubility and uptake of Fe in the rhizosphere. In the root cell plasma membrane, two different oxidoreductases capable of transferring electrons from the cytosol to several external electron acceptors (ferricyanide or ferric chelates) are involved in Fe acquisition. One oxidoreductase reduces only Fe(III) to ferricyanide, and the other is capable of reducing both ferric chelates and ferricyanide. This latter reductase, called Fe(III)-chelate reductase (FeCH-R), is induced or stimulated by iron-deficiency stress and is responsible for generating Fe(II) prior to uptake by dicotyledonous and nongraminaceous monocotyledonous plants [77]. Therefore, Fe uptake and thus the nutritional status of this micronutrient depends greatly on FeCH-R activity. It has been reported that some rootstocks have the potential to improve the Fe uptake through a higher FeCH-R activity. For instance, tomato and watermelon plants grafted onto *S. lycopersicum* variety "TmKnvf$_2$" and *C. maxima* variety "Dulce maravilla" rootstocks, respectively, exhibited a higher FeCH-R activity in the roots compared to ungrafted plants [78]. The higher FeCH-R activity in the roots of grafted watermelon plants was associated with a higher Fe content in leaves. Increases in FeCH-R activity is frequently observed in dicots cultivated under alkaline conditions, and this has been assumed to arise from an inducible plasma membrane-bound FeCH-R enzyme(s) [79–81]. However, no significant differences were observed in root FeCH-R activity between grafted and ungrafted watermelon plants grown under alkaline conditions, although the grafted watermelon plants onto *Cucurbita* hybrid rootstocks exhibited a higher leaf Fe concentration than ungrafted plants [67].

38.4
Heavy Metals and Excessive Amount of Trace Elements

38.4.1
Effects on Grafted Plants

38.4.1.1 **Growth and Yield**

Heavy metals such as cadmium, nickel, and chrome and excessive amount of trace minerals such as copper manganese and boron in soil and water are toxic to plants even at very low concentrations, or may accumulate in plant tissues up to a certain

level without visible symptoms or yield reduction [19, 82]. Rouphael *et al.* [19] have demonstrated that grafting cucumber onto the commercial rootstock "Shintoza" (*C. maxima* Duch. × *C. moschata* Duch.) mitigated the adverse effects of excessive Cu supply on plant biomass and fruit yield. In fact, shoot and root biomass weight reductions in control plants were clearly lower in grafted than in ungrafted plants, whereas the root to shoot ratio increased in ungrafted plants as a result of Cu stress conditions. Boron toxicity can also be mitigated by grafting onto suitable rootstocks, as indicated by an experiment with melon (*C. melo* L.) plants, which were exposed to five different B concentrations ranging from 0.1 to 10 mg L^{-1} in the irrigation water [34, 83]. The nongrafted melon plants were more sensitive to excess boron supply than the grafted ones (*C. maxima* Duch. × *C. moschata* Duch."TZ-148") in terms of fruit yield and dry weight accumulation in shoots and roots [34, 83].

38.4.1.2 **Fruit Quality**

Fruit vegetables are characterized by rather low rates of heavy metal and trace mineral translocation to the fruit [84]. However, contaminated vegetables are frequent in the market due to environmental pollution caused by human activities. For instance, a survey in Japan showed that approximately 7% of eggplant fruits contain cadmium concentrations above the international limit for fruiting vegetables. It was suggested that grafted plants could be used to prevent the entry of heavy metals and excessive amount of trace minerals into the supply chains via plants under unfavorable conditions [44]. Arao *et al.* [85] conducted a study to develop a method to reduce Cd concentration in eggplant fruits. They showed that grafting onto *S. torvum* reduced eggplant fruit Cd concentration by 63–75% in Cd-polluted soil and unpolluted soil compared to grafting onto *S. melongena* and *S. integrifolium*. The accumulation of Cu in fruit tissue of cucumber plants grown under Cu-enriched solution was significantly lower in plants grafted onto the "Shintoza"-type rootstock (*C. maxima* Duch. × *C. moschata* Duch.) in comparison to that of ungrafted plants [19]. Similarly, the concentrations of B, Zn, Sr, Mn, Cu, Ti, Cr, Ni, and Cd in the fruits of melon plants irrigated with marginal water were lower in the grafted plants onto the commercial *Cucurbita* rootstock "TZ-148" than in ungrafted plants [86].

38.4.2
Mechanisms of Tolerance in Grafted Plants

The enhanced tolerance of grafted vegetables to heavy metals and excessive amount of trace element has often been associated with root exclusion and/or to the restricted translocation from roots to shoots. Arao *et al.* [85] observed that grafting *S. melongena* plants onto *S. torvum* reduced the leaf and stem Cd concentrations by 67–73% in comparison to self-grafting or grafting onto *S. integrifolium*, in both Cd-polluted and unpolluted soils. The Cd concentration in xylem sap collected from stems of *S. torvum* was 22% of that in stems of *S. melongena*, indicating an appreciable restriction of the Cd translocation from root to shoot in the former. However, the concentrations of Cd in the roots of *S. melongena* and *S. torvum* were similar when the plants were exposed to identical external Cd levels [20]. These results indicate that *S. torvum* restricts

specifically the translocation of Cd to the shoot and not the Cd uptake by the roots. Genotypic differences in the ability of the root to prevent Cd translocation to the shoot have been reported also for soybean by means of grafting experiments [87]. According to Mori *et al.* [20], the restriction of Cd translocation to the fruit of eggplant grafted onto *S. torvum* in comparison to self-grafted *S. melongena* seems to be related to the process of xylem loading. Yamaguchi *et al.* [88] attempted to elucidate the molecular mechanisms governing the reduced Cd uptake by *S. torvum* and found that dehydration-related transcription factors and aquaporin isoforms are potential constituents of Cd-induced biochemical impediments. Other results have shown that the rootstock significantly affects gene expression in the scion, thereby indicating that some signals transported from the root to the shoot may also influence the Cd uptake and translocation [89]. Edelstein *et al.* [83] recorded that grafting melon onto the commercial *C. maxima* Duch. × *C. moschata* Duch. rootstock "TZ-148" reduced the boron concentration in the leaves of grafted plants in comparison to ungrafted plants. The lower boron concentration could be the result of mainly the differences in the properties of the root systems of the two plant types. Boron could accumulate and bind in the root system, which would limit its movement toward the shoot, as reported for some fruit trees [90, 91]. It should be noted, however, that in the current study boron concentrations in the root systems of both grafted and ungrafted melon plants were relatively low and, moreover, the boron concentrations in the roots of the ungrafted plants were similar to or higher than those in the roots of the grafted ones. Thus, it can be concluded that the lower boron concentrations in the leaves of the grafted plants than in those of the ungrafted ones were not the result of greater boron accumulation and attachment in the roots of the grafted plants than in those of the ungrafted ones [83]. Grafting cucumber cv. "Akito" onto the commercial rootstock "Shintoza" restricted the uptake and translocation of Cu to the shoot [19]. The leaf Cu concentration in grafted plants treated with a nutrient solution containing 47 and 94 μM Cu increased by 138 and 181%, respectively, in comparison to plants supplied with 0.3 μM Cu, while in ungrafted plants the increase in the leaf Cu level was 235 and 392%, respectively. Rouphael *et al.* [19] attributed the improved crop performance of grafted cucumber plants to the ability of the squash rootstock to restrict the accumulation of Cu in the shoot. These results indicate that Cu toxicity in cucumber cultivated in environments with too high Cu levels in the root zone may be partly mitigated by grafting onto the rootstock "Shintoza." Similarly, Savvas *et al.* [92, 93] found that the transport of Cu to the leaves of tomato "Belladona" was also restricted when the plants were grafted onto the "He-Man" rootstock (*S. lycopersicum* L. × *S. habrochaites* S. Knapp & D.M. Spooner). However, the concentration of Cu was significantly lower not only in the leaves but also in the roots of plants grafted onto "He-Man" in comparison to self-grafted "Belladona" plants.

38.5
Concluding Remarks and Future Perspectives

For decades, vegetable grafting has been successfully practiced in many Asian countries, and it is becoming increasingly popular in Europe as well. This chapter

concludes that grafting is an effective way to mitigate the detrimental effects of adverse soil chemical conditions such as salinity, alkalinity, heavy metals, and excessive amount of trace elements on vegetable crop performances particularly in Cucurbitaceae and Solanaceae. The increased tolerance of grafted plant to adverse soil chemical conditions was due to the use of tolerant rootstocks. Several effective rootstocks are mentioned and already in practical use, or used in breeding programs. The mechanisms involved in the advantageous response of specific stress-tolerant rootstocks are manifold and partly still unknown. Augmentation of this knowledge may help to select and breed appropriate rootstocks that improve the adaptability of fruit vegetable crops to salinity, alkalinity, heavy metals, and excessive amount of trace elements. The agronomical and physiological processes implicated in the tolerance of grafted plants to adverse soil chemical conditions have received much attention, but the biochemical and molecular processes involved remain relatively unknown. So, a thorough investigation should be conducted with the aim of providing biochemical and molecular knowledge on the metabolism of grafted plants grown under adverse soil chemical conditions. Finally, researchers, extension specialists, and seed companies need to work together to integrate this modernized technology as an effective tool for producing high-quality vegetables under adverse soil chemical conditions.

References

1 Munns, R. (2005) *New Phytol.*, **167**, 645–663.
2 Mohamed, A.A., Agnolon, F., Cesco, S. *et al.* (1998) *Agrochimica*, **XLII** (6), 255–262.
3 Darwish, T., Atallah, T., Moujabber, M.E., and Khatib, N. (2005) *Agric. Water Manage.*, **78**, 152–164.
4 Yu, H.Y., Li, T.X., and Zhou, J.M. (2005) *Soils*, **37**, 581–586.
5 Grattan, S.R. and Grieve, C.M. (1999) *Sci. Hort.*, **78**, 127–157.
6 Munns, R. and Tester, M. (2008) *Annu. Rev. Plant Biol.*, **59**, 651–681.
7 Marschner, H. (1995) Adaptation of plants to adverse chemical soil conditions, in *Mineral Nutrition of Higher Plants*, 2nd edn (ed. H. Marschner), Academic Press, London, pp. 596–680.
8 Misra, A. and Tyler, G. (1999) *Ann. Bot.*, **84**, 401–410.
9 Pissaloux, A., Morarad, P., and Bertoni, G. (1995) Alkalinity: bicarbonate calcium effects on iron chlorosis in white lupine in soilless culture, in *Iron Nutrition in Soils and Plants* (ed. J. Abadía), Seventh International Symposium on Iron Nutrition and Interactions in Plants, Zaragoza, Spain, June 27–July 2, 1993 Kluwer Academic Publishers, Dordrecht, pp. 127–133.
10 Mengel, K. (1994) *Plant Soil*, **165**, 275–283.
11 Gogorcena, Y., Abadía, J., and Abadía, A. (2004) *J. Plant Nutr.*, **27**, 1701–1715.
12 Nedunchezhian, N., Morales, F., Abadía, A., and Abadía, J. (1997) *Plant Sci.*, **129**, 29–38.
13 Amman, A.A., Michalke, B., and Schramel, P. (2002) *Anal. Biochem.*, **372**, 448–452.
14 Ross, S.M. (1994) *Toxic Metals in Soil–Plant Systems*, John Wiley & Sons, Ltd., Chichester, UK.
15 Kar, D., Sur, P., Mandal, S.K. *et al.* (2008) *Int. J. Environ. Sci. Tech.*, **5**, 119–124.
16 Zheng, Y., Wang, L., and Dixon, M. (2005) *HortScience*, **40**, 2132–2134.
17 Burzynski, M. and Klobus, G. (2004) *Photosynthetica*, **42**, 505–510.
18 Wang, S.H., Yang, Z.M., Yang, H. *et al.* (2004) *Bot. Bull. Acad. Sin.*, **45**, 203–212.

19 Rouphael, Y., Cardarelli, M., Rea, E., and Colla, G. (2008) *Environ. Exp. Bot.*, **63**, 49–58.
20 Mori, S., Uraguchi, S., Ishikawa, S., and Arao, T. (2009) *Environ. Exp. Bot.*, **67**, 127–132.
21 Rus, A.M., Estan, M.T., Gisbert, C. *et al.* (2001) *Plant Cell Environ.*, **24**, 875–880.
22 Bonhert, H.J. and Jensen, R.G. (1996) *Aust. J. Plant Physiol.*, **23**, 661–666.
23 Lee, J.M., Kubota, C., Tsao, S.J. *et al.* (2010) *Sci. Hort.*, **127**, 93–105.
24 Louws, F.J., Rivarda, C.L., and Kubota, C. (2010) *Sci. Hort.*, **127**, 127–146.
25 Maas, E.V. (1986) *Appl. Agr. Res.*, **1**, 12–36.
26 Martinez-Rodriguez, M.M., Estañ, M.T., Moyano, E. *et al.* (2008) *Environ. Exp. Bot.*, **63**, 392–401.
27 Estañ, M.T., Martinez-Rodriguez, M.M., Perez-Alfocea, F. *et al.* (2005) *J. Exp. Bot.*, **56**, 703–712.
28 Liu, Z.L., Zhu, Y.L., Wei, G.P. *et al.* (2007) *Acta Bot. Boreal-Occident Sin.*, **27**, 1795–1800.
29 Wei, G.P., Zhu, Y.L., Liu, Z.L. *et al.* (2007) *Acta Bot. Boreal-Occident. Sin.*, **27**, 1172–1178.
30 Goreta, S., Bucevic-Popovic, V., Selak, G.V. *et al.* (2008) *J. Agri. Sci.*, **146**, 695–704.
31 Yetisir, H. and Uygur, V. (2010) *J. Plant Nutr.*, **33**, 315–327.
32 Huang, Y., Zhu, J., Zhen, A. *et al.* (2009) *J. Food Agric. Environ.*, **7**, 703–708.
33 Romero, L., Belakbir, A., Ragala, L., and Ruiz, J.M. (1997) *Soil Sci. Plant Nutr.*, **43**, 855–862.
34 Edelstein, M., Ben-Hur, M., Cohen, R. *et al.* (2005) *Plant Soil*, **269**, 273–284.
35 Colla, G., Rouphael, Y., Cardarelli, M. *et al.* (2006) *J. Hortic. Sci. Biotechnol.*, **81**, 146–152.
36 Huang, Y., Bie, Z.L., He, S.P. *et al.* (2010) *Environ. Exp. Bot.*, **69**, 32–38.
37 Colla, G., Rouphael, Y., Cardarelli, M., and Rea, E. (2006) *HortScience*, **41**, 622–627.
38 Yang, L.F., Zhu, Y.L., Hu, C.M. *et al.* (2006) *Acta. Bot Boreal-Occident. Sin.*, **26**, 1195–1200.
39 He, Y., Zhu, Z.J., Yang, J. *et al.* (2009) *Environ. Exp. Bot.*, **66**, 270–278.
40 Moya, J.L., Primo-Millo, E., and Talon, M. (1999) *Plant Cell Environ.*, **22**, 1425–1433.
41 Karaba, A., Dixit, S., Greco, R. *et al.* (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 15270–15275.
42 Santa-Cruz, A., Martínez-Rodríguez, M.M., Cuartero, J., and Bolarin, M.C. (2001) *Acta Hortic.*, **559**, 413–417.
43 Francois, L.E. and Maas, E.V. (1994) Crop response and management of salt-affected soils, in *Handbook of Plant and Crop Stress* (ed. M. Pessarakli), Marcel Dekker, New York, pp. 449–459.
44 Rouphael, Y., Schwarz, D., Krumbein, A., and Colla, G. (2010) *Sci. Hort.*, **127**, 172–179.
45 Gomez, J.M., Hernandez, J.A., Jimez, A. *et al.* (1999) *Free Rad. Res.*, **3**, 11–18.
46 Fernández-García, N., Martínez, V., Cerda, A., and Carvajal, M. (2004) *J. Hortic. Sci. Biotechnol.*, **79**, 995–1001.
47 Levander, O.A. (1990) *HortScience*, **25**, 1486–1488.
48 Zhu, J., Bie, Z.L., Huang, Y., and Han, X.Y. (2008) *Soil Sci. Plant Nutr.*, **54**, 895–902.
49 Apse, M.P., Aharon, G.S., Snedden, W.A., and Blumwald, E. (1999) *Science*, **285**, 1256–1258.
50 Tester, M. and Davenport, R.J. (2003) *Ann. Bot.*, **91**, 503–527.
51 Chen, S.F., Zhu, Y.L., Zhang, G.W. *et al.* (2008) *Plant Nutr. Fert. Sci.*, **14**, 1098–1103.
52 Edelstein, M., Plaut, Z., and Ben-Hur, M. (2011) *J. Exp. Bot.*, **62**, 177–184.
53 Zhu, J.K. (2001) Plant salt stress. *ELS*. doi: 10.1002/9780470015902.a0001300.pub2
54 Huang, Y., Bie, Z.L., Liu, Z.X. *et al.* (2009) *Soil Sci. Plant Nutr.*, **55**, 698–704.
55 Apel, K. and Hirt, H. (2004) *Annu. Rev. Plant Biol.*, **55**, 373–399.
56 Ashraf, M. (2009) *Biotechnol. Adv.*, **27**, 84–93.
57 Ghanem, M.E., Albacete, A., Smigocki, A.C. *et al.* (2011) *J. Exp. Bot.*, **62**, 125–140.
58 Albacete, A., Martínez-Andújar, C., Ghanem, M.E. *et al.* (2009) *Plant Cell Environ.*, **32**, 928–938.
59 Liu, H.P., Dong, B.H., Zhang, Y.Y. *et al.* (2004) *Plant Sci.*, **166**, 1261–1267.
60 Chen, S.F., Zhu, Y.L., Liu, Y.L. *et al.* (2006) *Acta Hortic. Sin.*, **33**, 58–62.
61 Liu, Z.L., Zhu, Y.L., Wei, G.P. *et al.* (2008) *Acta Ecol. Sin.*, **28**, 1586–1592.
62 Xu, S.L., Chen, Q.Y., Chen, X.Q., and Li, S.H. (2006) *J. Fruit Sci.*, **23**, 260–265.

63 Alhendawi, R.A., Römheld, V., Kirkby, E.A., and Marschner, H. (1997) *J. Plant Nutr.*, **20**, 1731–1753.

64 Campbell, S.A. and Nishio, J.N. (2000) *J. Plant Nutr.*, **23**, 741–757.

65 Zribi, K. and Gharsalli, M. (2002) *J. Plant Nutr.*, **25**, 2143–2149.

66 Valdez-Aguilar, L.A. (2004) Effect of alkalinity in irrigation water on selected greenhouse crops. PhD thesis in Horticulture, Texas A&M University, 500014, p. 489.

67 Colla, G., Rouphael, Y., Cardarelli, M. *et al.* (2010) *Environ. Exp. Bot.*, **68**, 283–291.

68 Römheld, V. and Marschner, H. (1986) *Adv. Plant Nutr.*, **2**, 155–204.

69 Gharsalli, M., Zribi, K., and Hajji, M. (2001) Physiological responses of pea to iron deficiency induced by bicarbonate, in *Plant Nutrition: Food Security and Sustainability of Agro-Ecosystems* (ed. W.J. Horst *et al.*), Kluwer Academic, the Netherlands, pp. 606–607.

70 Gent, M.P.N., Parrish, Z.D., and White, J.C. (2005) *J. Am. Soc. Hort. Sci.*, **130**, 782–788.

71 Kraffczyk, I., Trolldenier, G., and Beringer, H. (1984) *Soil Biol. Biochem.*, **16**, 315–322.

72 Zhang, F., Römheld, V., and Marschner, H. (1989) *Zeit. Pflanz. Bodenk.*, **152**, 205–210.

73 Nielsen, N.E. (1976) *Plant Soil*, **45**, 679–687.

74 Jones, D.L. and Darrah, P.R. (1994) *Plant Soil*, **166**, 247–257.

75 Gerke, J., Römer, W., and Jungk, A. (1994) *Zeit. Pflanz. Bodenk.*, **157**, 289–294.

76 Jauregui, M.A. and Reisenauer, H.M. (1982) *Soil Sci. Soc. Am. J.*, **46**, 314–317.

77 Marschner, H. and Römheld, V. (1995) Strategies of plants for acquisition of iron, in *Iron Nutrition in Soils and Plants* (ed. J. Abadía), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 375–388.

78 Rivero, R.M., Ruiz, J.M., and Romero, L. (2004) *J. Plant Nutr.*, **27**, 2221–2234.

79 Bienfait, H.F. (1988) *Plant Physiol.*, **88**, 785–787.

80 Bagnaresi, P. and Basso, B. (1996) *J. Plant Nutr.*, **19**, 1171–1179.

81 Brüggemann, W. and Moog, P.R. (1989) *Physiol. Plant.*, **75**, 245–254.

82 Verkleij, J.A.C., Golan-Goldhirsh, A., Antosiewisz, D.M. *et al.* (2009) *Environ. Exp. Bot.*, **67**, 10–22.

83 Edelstein, M., Ben-Hur, M., and Plaut, Z. (2007) *J. Am. Soc. Hort. Sci.*, **132**, 484–491.

84 Angelova, V.R., Babrikov, T.D., and Ivanov, K.I. (2009) *Commun. Soil Sci. Plant Anal.*, **40**, 2248–2263.

85 Arao, T., Takeda, H., and Nishihara, E. (2008) *Soil Sci. Plant Nutr.*, **54**, 555–559.

86 Edelstein, M. and Ben-Hur, M. (2006) *Acta Hortic.*, **699**, 159–167.

87 Sugiyama, M., Ae, N., and Arao, T. (2007) *Plant Soil*, **295**, 1–11.

88 Yamaguchi, H., Fukuoka, H., Arao, T. *et al.* (2010) *J. Exp. Bot.*, **61**, 423–437.

89 Si, Y., Dane, F., Rashotte, A. *et al.* (2010) *J. Exp. Bot.*, **61**, 1635–1642.

90 Elmotaium, R., Hu, H.N., and Brown, P.H. (1994) *J. Am. Soc. Hort. Sci.*, **119**, 1169–1175.

91 Papadakis, I.E., Dimassi, K.N., Bosabalidis, A.M. *et al.* (2004) *Plant Sci.*, **166**, 539–547.

92 Savvas, D., Papastavrou, D., Ntatsi, G. *et al.* (2009) *HortScience*, **44**, 1978–1982.

93 Savvas, D., Colla, G., Rouphael, Y., and Schwarz, D. (2010) *Sci. Hort.*, **127**, 156–161.

39
Grain Legumes (Soybean, Chickpea, and Peanut): Omics Approaches to Enhance Abiotic Stress Tolerance

D. Srinivas Reddy, Pooja Bhatnagar-Mathur, Vincent Vadez, and Kiran K. Sharma

Legumes rank third in world crop production, and abiotic stress is the major constraint to crop productivity. Biotechnological applications including all "omics" have been the direct and potential approaches for improving abiotic stress tolerance in grain legumes and requires knowledge of stress response at molecular level, which includes gene expression to protein or metabolite and its phenotypic effects. Genome-wide expression profiling studies have been carried out in the legumes to identify the candidate genes and regulatory networks among abiotic stress responses. Among the grain legumes, although soybean has been more intensively studied, more recently, sensitive and tolerant varieties of chickpea and peanut have been characterized under abiotic stress conditions. Nevertheless, proteomic studies in response to abiotic stress in legumes are still very limited with only *Medicago truncatula* and soybean protein reference maps available. Some of the major QTL controlling abiotic stress tolerance in legumes have been mapped for a major QTL for salt tolerance in soybean and drought tolerance-related traits in peanut. Although, *Agrobacterium*-mediated gene transfer has been reported in all the major legume crops, so far only one legume, that is, soybean, has been commercialized. Transgenic technologies for improved abiotic stress tolerance involving regulatory genes have proved more efficient than using single or multiple functional genes involved in stress tolerance. Hence, the current advances in "omics" technologies and availability of the genome sequences of model legumes and soybean offer great potential to improve the stress tolerance of the legume crops. This chapter attempts to provide a detailed discussion about the different "omics" approaches and their applications for abiotic stress research on major legumes.

39.1
Introduction

Legumes represent the most utilized plant family with 20 000 species and are among the most important crops worldwide, having major impacts on agriculture, the

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environment, and human/animal nutrition and health [1]. Legumes rank third behind cereals and oilseeds in world production [2] that accounts for 27% of the world's primary crop production [1]. Grain legumes constitute an important dietary constituent for humans and animals and these alone contribute 33% of the dietary protein nitrogen (N) needs of humans [3] besides being a source of income and livestock feed. These perfectly match the requirements of small-scale, low-income farmers in the developing countries where they accounted for 61.3 million hectares in 2002, compared to 8.5 million hectares in developed countries [2]. In order of rank, common beans (*Phaseolus* spp.), pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), broad bean (*Vicia faba* L.), pigeonpea (*Cajanus cajan* L.), cowpea (*Vigna unguiculata* L.), and lentil (*Lens esculentum* L.) constitute the primary dietary legumes [4]. Moreover, grain legumes, predominantly soybean (*Glycine max* L.) and peanut (*Arachis hypogeae* L.), are also a major source for vegetable oil, providing more than 35% of the world's processed vegetable oil.

Abiotic stress is the major constraint to crop productivity in the semiarid tropics (SAT) that include parts of 55 developing countries, populated by about 1.4 billion people, where grain legumes are mainly cultivated. Abiotic stress, which includes multiple stresses such as drought, salinity, waterlogging, high temperature, chilling, and so on are the primary causes of crop losses worldwide, reducing average yields for most major crop plants by over 50% [5, 6]. Only 10% of the global arable land can be classified under the nonstress category, which implies that crops grown on the other 90% of arable lands experience one or more environmental stresses [7]. Furthermore, crops under abiotic stress are usually more susceptible to weeds, insects, and diseases, which considerably increase the losses [8].

The grain legumes constitute important food and oilseed crops of the SAT, are mostly grown in low-input, rain-fed agriculture, and suffer from drought due to insufficient, untimely, and erratic rainfall in these climates that becomes major constraints to crop productivity. Several of the abiotic stresses associated with legume crops also directly affect symbiotic interactions and therefore limit their growth. Water deficits continue to be the major abiotic factor that affect crop yields globally [9] and are likely to worsen with the projected rapid expansion of water-stressed areas of the world encompassing 3 billion people by 2030 [10]. Moreover, in legumes such as peanut (*A. hypogaea*), Brazil nuts (*Bertholletia excelsa*), and faba bean (*V. faba*), aflatoxin contamination is a common occurrence during preharvest drought stress [11, 12]. In addition to drought, soil salinity is another major problem affecting the total nitrogen uptake and soil nitrogen contribution [13] resulting in reduced yields. Hence, there is a crucial need to increase the abiotic stress tolerance in legumes, which is a major challenge in crop improvement programs for enhancing yield stability. Although conventional plant breeding and enhanced management strategies have addressed several constraints that limit crop productivity or quality, there are situations where the existing genetic resources lack the required traits. Yield losses due to constraints like drought are highly variable in nature depending on the stress timing, intensity, and duration. Moreover, location-specific environmental stress factors such as high irradiance and temperature make breeding for drought

tolerance difficult through conventional approaches. Cutting-edge, knowledge-based breeding practices complemented adequately by genomics and genetic transformation technologies could lead to simpler and more effective gene-based approach for improving abiotic stress tolerance in the grain legumes. Application of biotechnological approaches has a potential to contribute efficiently to solve or reduce these problems in the grain legumes, thereby contributing to sustainable agriculture, especially in the SAT.

39.2 "OMICS" in Legumes and Abiotic Stress

Biotechnological approaches such as tissues culture, *in vitro* mutagenesis, marker-assisted breeding, and genetic transformation can speed up and overcome major bottlenecks of classical plant breeding due to the lack of natural sources of resistance and sexual incompatibility. However, successful application of biotechnology to abiotic constraints requires a good biological knowledge of both the target species and the mechanisms underlying tolerance to these stresses. Mechanisms of responses to stress can be measured at many different levels from the whole plant to the molecular level. The type, length, and severity of the stress have more influence on the plant response to stress [14]. Since responses are controlled by the plant genome, recent efforts have focused on the molecular response of the plant to water deficits [15]. Until a few years, the research on plant stress responses was focused on model plants such as *Arabidopsis*, and not much work was done on the legumes. However, since substantial similarities exist between the two crops, the knowledge on stress responses of *Arabidopsis* were used as source of information for legume research. Nevertheless, there are also significant fundamental differences like all physiological processes that differ and must be exploited to unravel the specific mechanisms involved in abiotic stress tolerance in the legumes [16]. Since the large genome size and the polyploidy of some legumes have hampered this goal, recent progress in legume biology has been greatly enhanced by the development of model systems to investigate the genetics of nodulation and other important processes such as resistance or tolerance to stresses. The two model legume plant systems, *Lotus japonicus* and *Medicago truncatula*, due to their small and diploid genomes, autogamous nature, short generation times, and prolific seed production were the obvious choices [17, 18]. Since then, powerful genetic and genomic tools have been developed that include genome sequencing [19], isolation of expressed sequence tags (ESTs) [20, 21], and establishment of genetic and physical maps for each model species [22, 23]. The increasing wealth of genetic and genomic data and the high degree of synteny between legume genomes [24, 25] make these two species valuable models for the molecular genetic study of the biotic and abiotic constraints that hamper legume crop yields. Furthermore, the soybean genome sequence and the high synteny between soybean and the model legumes have a potential to facilitate positional cloning and other genetic procedures for these studies.

While sequence information is invaluable and a necessary starting point, it is insufficient to answer questions concerning gene function, regulatory networks, and the biochemical pathways activated in response to stresses. To address these questions, more comprehensive approaches, including quantitative and qualitative analyses of gene expression products are necessary at the transcriptomic, proteomic, and metabolomic levels. This comprehensive knowledge about the genes involved in stress response and tolerance will further allow a more precise use of marker-assisted selection (MAS) and transgenics [7]. Since the "omics" involves genomics and functional genomics, genetic engineering, transcriptome profiling, proteomics, and metabolomics describing an organism's genome contribution to its overall phenotype, the recent progress made in these areas has considerably contributed to better understanding of the molecular and genetic basis of stress response that has been an important bottleneck for molecular and transgenic breeding. So far, a significant progress has been made in research on the abiotic stress tolerance of major legumes including soybean, chickpea, and peanut as discussed in the following sections.

39.3 Transcript "OMICS"

A eukaryotic cell contains ~15 000–30 000 distinct mRNAs with a prevalence ranging from one to several thousands in a total mass of ~100 000 mRNAs [26]. About 50% of the transcript population is made up of a relatively small number (some hundreds) of abundant transcripts representing only 1% of the different mRNA species, and the other half contains the "rare" mRNAs [27]. The set of all the messenger RNAs (mRNAs) in a cell/tissue/organism is referred to as the transcriptome and investigation of populations of mRNAs is thus called "transcriptomics." A genome-wide expression profiling is a powerful tool for studying genes involved in various biological phenomena, identifying the candidate genes, and revealing the molecular crosstalk of gene regulatory networks among abiotic stress responses.

Plants undergoing abiotic stresses in general face dehydration at the cellular level and hence almost 50% of the genes activated by these stresses including drought, salinity, or ABA treatment are common. Cellular water deficit in a plant stress triggers many changes in gene expression that in turn define its response to a particular environmental condition. The induced genes in response to cellular water deficit stress constitute different functional categories such as metabolism, transport, signaling, transcription, hydrophilic proteins, and the unknown, including the repression of genes involved in plant growth and development, such as photosynthesis-related genes. Broadly, the genes responding to abiotic stress can be categorized into two classes based on their response in terms of timescale or based on their involvement in tolerance; some respond immediately within seconds or minutes, while others respond later, in hours, days, or even weeks [28]. This allows for the speculation that the early responsive genes may provide initial protection and

regulate gene expression by being involved in amplification of signals and signal transduction. These include various protein kinases and genes encoding transcription factors, whereas the genes that respond later may be involved in adaptation to stress conditions, such as heat shock proteins, LEA proteins, ROS scavenger proteins, and so on [14, 28].

The genomic approaches allow changes induced by abiotic stresses on a global scale to be analyzed at the level of the whole organism. Much more extensive gene expression studies have been performed in *Arabidopsis*, and the resulting knowledge can also be used in legumes through comparative genomics. For example, Ishitani *et al*. [29] selected 100–200 genes from the *Arabidopsis* database and showed that at least 3 DREB-like genes, thought to be key transcriptional regulators of drought and/or cold tolerance, were present in common bean. Similarly, in *Arabidopsis*, analysis of the transcriptome changes occurring during cold, drought, and salt stress in a survey of 7000 genes showed a shared response for a majority of cold and drought stress-regulated genes, supporting the hypothesis that a common set of signal transduction pathways are triggered during different stress responses [30]. Around 11% of the stress-inducible genes are potential transcription factors further confirming the relevance of gene regulation in stress adaptation [31].

The *Arabidopsis* model is likely to be very different from legumes in terms of responses to stress in relation to grain filling, nitrogen utilization, fixation, and transport, root architecture, and interactions, all physiological processes that are fundamentally different in legumes. Hence, the usefulness of developing a legume model has become increasingly relevant in recent years. Moreover, the induction of gene expression by environmental stress must be exploited to unravel mechanisms dealing with abiotic stress tolerance in the agriculturally important grain legumes. In legumes, the gene expression patterns following biotic stresses have been more extensively studied than those following abiotic stresses. With respect to abiotic stress, gene expression analyses have been mainly based on studies with cloned genes [32]. Significant progress is being made at the genetic and genomic levels using the model legume *M. truncatula* through macro- and microarray analysis, reverse genetics, genome sequencing, and other high-throughput techniques [33, 34]. The analysis of almost 200 000 ESTs of *M. truncatula*, isolated from many different libraries constructed from diverse stages and treatments, was facilitated by searchable databases such as MtDB2 [35] and the TIGR Gene Index (http://www.tigr.org).

The advent of next-generation sequencing platforms [36], most recently the "third generation" (also called "next–next generation" or NGS) sequencing systems will enable plant genome to be sequenced within hours. The NGS approaches allow deciphering the cell's transcripts on the sequence level, which will truly revolutionize the research of organisms that are not now in line for genomic sequencing. This approach could circumvent the problems posed by extremely large genomes such as legumes. The next-generation sequencing not only is a dramatic advance over capillary-based sequencing but also presents significant challenges in assembly and sequence accuracy due to short read lengths, method-specific sequencing errors, and the absence of physical clones. However, the promise of much lower sequencing cost

with the now proven concept of next-generation expressed sequence tag sequencing will allow assessment of plant genomes at least at the functional level [37]. At ICRISAT, these NGS approaches are being used to develop EST-based markers to map the QTL for stress response in grain legumes. Recent reports have also shown that transcriptomic tools are a good option for legume breeding to environmental stresses as discussed in the next sections.

39.3.1 Soybean

Among the grain legumes, soybean has been more intensively studied and according to the legume information system data, over 1.3 million ESTs were developed from different cDNA libraries, which is the largest in number among the individual grain legume ESTs. The availability of a large number of EST and BAC sequences facilitated the discovery of new SNP and SSR markers in soybean toward the construction of high-resolution genetic maps. Besides, using a modified cDNA-AFLP technique in soybean, 140 differentially expressed cDNA fragments were obtained by comparing control and isoosmotic treated plants where some of the responsive genes encoded for ion transporters, transcription factors (TFs), and redox enzymes [38].

39.3.2 Chickpea

Chickpea is the most important food legume of semiarid tropics (SAT) and taxonomically one of the closest crops to the model legume *Medicago*. Sensitive and tolerant varieties of chickpea have been characterized under abiotic stress conditions, although very little is known about the genes involved in these responses. However, the characterization of genes involved in the differential behavior of these cultivars may constitute a good basis to extrapolate these results to other grain legumes. Five differentially expressed cDNAs were identified using differential display reverse transcriptase PCR (DDRT-PCR) under drought conditions with drought-tolerant cv. ICCV2 and drought-susceptible cv. ILC3279 of chickpea [39]. Moreover, 319 unique ESTs available from different libraries have been analyzed for differences in transcript profiling during drought stress treatment in two chickpea varieties having contrasting levels of drought tolerance (*C. arietinum* cv. PUSABGD72 and ICCV2). These ESTs were clustered in four groups according to their expression patterns [40].

A transcriptional profiling study in chickpea under drought, cold, and high salinity was carried out using cDNA microarray approach to look at the gene expression in the leaf, root, and/or flower tissues in tolerant and susceptible genotypes [41]. The differentially expressed transcripts in response to the particular stress were analyzed and a transcriptional change of over twofold was observed for 109, 210, and 386 genes after drought, cold, and high-salinity treatments, respectively. Among these, 2, 15, and 30 genes were consensually differentially expressed between tolerant and

susceptible genotypes studied for drought, cold, and high salinity, respectively. The differentially expressed genes coded for various functional and regulatory proteins, highlighting the multiple gene control and complexity of abiotic stress response mechanism in chickpea.

Two nonnormalized cDNA libraries from the seedling leaves of a drought-tolerant chickpea cultivar under PEG-treated and nontreated conditions have been constructed where 92 differentially expressed genes were identified [42]. Most of the upregulated genes were related to drought tolerance, while the downregulated genes were mainly involved in the photosynthesis. A set of over 2800 chickpea ESTs have been generated from a library constructed after subtractive suppressive hybridization (SSH) of root tissue from two closely related chickpea genotypes possessing different sources of drought avoidance and tolerance, ICC4958 (tester) and Annigeri (driver), respectively [43]. A total of 106 EST-based markers were designed from 477 sequences with functional annotations that were tested on *C. arietinum*. Forty-four EST markers were polymorphic when screened across nine *Cicer* species (including the cultigen) [44]. The chickpea root EST database developed in these studies provide researchers with a major new resource for data mining associated with root traits and drought tolerance [43]. More recently, a total of 20 162 drought- and salinity-responsive ESTs were generated from 10 different root tissue cDNA libraries of chickpea and 177 new EST-based SSR markers were developed [45].

Besides, SuperSAGE analysis for gene expression in chickpea roots in response to drought was carried out resulting in sequencing of 80 238 of 26 bp tags [46]. Among these tags, 7532 (43%) UniTags were more than 2.7-fold differentially expressed and 880 (5.0%) were regulated more than 8-fold upon stress resulting in unambiguous annotation of 22% (3858) of these tags. Microarray analysis of these 3000 annotated UniTags confirmed 79% of the tag-based results, whereas RT-PCR confirmed the SuperSAGE data in all cases. This is the first study to prove the potential of SuperSAGE technology for molecular breeding in the nonmodel crops. However, lack of availability of a chickpea reference genome limits the value of SuperSAGE tags, as only a fraction of them could be annotated.

39.3.3 Peanut

In peanut, differential DDRT-PCR has been used to identify differentially expressed genes in peanut grown under drought stress versus irrigation conditions where some drought-responsive mRNA transcripts were identified based on expression pattern [47, 48]. Besides, DDRT-PCR studies have been carried out with transgenic peanut events overexpressing rd29A:DREB1A to detect the differentially expressed transcripts under abiotic stress [49]. Here, 51 differentially expressed transcripts were identified under stress treatments; among them 35 transcripts were newly expressed, 11 were upregulated, and 5 were downregulated. In the BLAST search of differentially expressed partial cDNAs, only 17 clones showed a significant similarity to the ESTs in the database, indicating that the majority of the cDNAs cloned in this study may be novel and needs further research to identify their role in stress response. These

results also suggested that the increased plant tolerance against drought stress in transgenic peanut may not be attributable only to the expression of DREB1A-targeted cold-responsive (COR) genes identified in *Arabidopsis* [49].

In a recent study, six different cDNA libraries were constructed from developing peanut seeds at three reproduction stages (R5, R6, and R7) from a resistant and a susceptible cultivated peanut genotype, "Tifrunner" that is susceptible to *Aspergillus* infection with higher aflatoxin contamination and resistant to *tomato spotted wilt virus* (TSWV) and "GT-C20" that is resistant to *Aspergillus* with reduced aflatoxin contamination and susceptible to TSWV. The developing peanut seed tissues of these genotypes were challenged by *Aspergillus parasiticus* and drought stress in the field and 21 777 high-quality EST sequences were generated from cDNA clones of 6 libraries [50]. Similarly, EST libraries for cultivated peanut were developed from leaves of peanut line C34-24 (resistant to leaf spots and TSVW) and immature pods of peanut line A13 (tolerant to drought stress and preharvest aflatoxin contamination). A total of 1825 ESTs, 769 from the C34-24 and 1056 from the ESTs were identified and 44 EST-derived simple sequence repeat (SSR) markers have been characterized for cultivated peanut [51]. A total of 6264 high-quality ESTs were generated from leaves and roots of a wild peanut *Arachis stenosperma*, and 188 microsatellite markers have developed form these ESTs [52].

More recently, nearly 700 genes were identified in subtractive cDNA library from gradual process of drought stress adaptation in peanut. This study also showed the functional importance of HSP70 gene and key regulators such as Jumonji in drought stress response [53]. A high-density oligonucleotide microarray for peanut has also been developed using 49 205 publicly available ESTs and tested the utility of this array for expression profiling in a variety of peanut tissues [54]. Over 108 putatively pod-specific/abundant genes, as well as transcripts, whose expression was low or undetected in pod compared to peg, leaf, stem, or root were detected. Several transcripts that significantly overrepresented in the peanut pod included genes responsible for seed storage proteins and desiccation (e.g., late-embryogenesis abundant proteins, aquaporins, legumin B), oil production, and cellular defense [54].

39.4 Prote"omics"

Since the 1990s, genomics has been the most active research field in biological science generating a huge amount of information, while structural genomics has emerged at the methodological level to understand gene expression and function. A complete knowledge of the proteins expressed by the genome of a cell, tissue, or organism at a specific time point (proteome) is necessary to understand the biology of a cell or an organism. The proteome reflects the actual state of the cell or the organism and is an essential bridge between the transcriptome and the metabolome. Proteins act directly on biochemical processes, and thus must be closer to the phenotype, compared to DNA-based markers. Although research on plant responses to stress on the DNA or RNA level provided an important insight into stress tolerance, the

proteomics approach is very important in evaluating stress responses since the mRNA levels may not always correlate with protein accumulation [55]. In addition, many proteins are modified by posttranslational modifications such as phosphorylation, glucosylation, and ubiquitinylation, which significantly influence protein functions. Proteomics, understood as protein biochemistry on an unprecedented and high-throughput scale, is becoming a promising and active approach in this postgenomic period. However, its application to plants is rather limited compared to other biological systems [56].

Compared to analysis of the transcriptome, analysis of the plant proteome in response to abiotic and biotic stresses is still limited, although good technical progress has been achieved in the separation of proteins and their identification by mass spectrometry. Studies have evaluated changes in protein levels in plant tissues in response to stresses [57, 58]. However, these studies have mainly focused on nonlegume species such as *Arabidopsis* and rice [57] and some legumes recently [56]. As a result, only a handful of studies have been carried out in legumes, although in the next few years there should be a significant increase in the number of legume species and stresses analyzed. So far, pea has been more intensively studied, with the analysis of induced protein expression in roots in response to salt [59] and to cadmium stress [60]. Recently, *M. truncatila* has been the subject of several proteomic studies that represent the most extensive proteomic description of *M. truncatula* suspension cells to date and provide a reference map for future comparative proteomics and functional genomics studies of biotic and abiotic stress responses [61].

39.4.1 Soybean

Some reference maps of soybean that are available in the proteomics database provide a starting point for ongoing functional genomics studies associated with biotic/abiotic stress in soybean. The Soybean Proteome Database is aimed to be a data repository for functional analyses of soybean responses to flooding injury that is recognized as a major constraint for the establishment and production of this plant. The latest release contains 21 reference maps of soybean (*G. max* cv. Enrei) proteins electrophoresized on two-dimensional polyacrylamide gels of which the samples were collected from several organs, tissues, and organelles. These reference maps included 7311 detected proteins and 532 identified proteins, or proteins for which a sequence or peptide peak has been determined. The Soybean Proteome Database also integrates multiple "omes," where an "omics" table reveals relationships among 106 mRNAs, 51 proteins, and 89 metabolites that vary over time under flooding stress. The tabulated metabolites are anchored to a metabolome network. A unified temporal profile tag attached to the mRNAs, proteins, and metabolites facilitates retrieval of the data based on the temporal expression profiles. A graphical user interface based on dynamic HTML facilitates viewing of both the metabolome network and the profiles of multiple "omes" in a uniform manner. The entire database is available at http://proteome.dc.affrc.go.jp/soybean/ [62].

39.4.2 Chickpea

Most of the earlier understanding of dehydration-responsive cellular adaptation in chickpea has evolved from transcriptome analysis and the comparative analysis of dehydration-responsive proteins, particularly proteins in the subcellular fraction, is limited. Bhushan *et al.* [63] have initiated a proteomics approach to identify dehydration-responsive ECM proteins in JG-62, a drought-tolerant variety of chickpea where the dehydration-responsive temporal changes in ECM proteins revealed 186 proteins with variance at a 95% significance level. The comparative proteomics analysis led to the identification of 134 differentially expressed proteins that include predicted and novel dehydration-responsive proteins. This study, for the first time, demonstrated that over a 100 ECM proteins are presumably involved in a variety of cellular functions, namely, cell wall modification, signal transduction, metabolism, and cell defense and rescue, and impinge on the molecular mechanism of dehydration tolerance in plants. Since the nuclear proteins constitute a highly organized, complex network that plays diverse roles during cellular development and other physiological processes. Another study provided insights into the complex metabolic network operating in the nucleus during dehydration in chickpea [64]. Approximately, 205 protein spots were found to be differentially regulated under dehydration; mass spectrometry analysis allowed the identification of 147 differentially expressed proteins, presumably involved in a variety of functions including gene transcription and replication, molecular chaperones, cell signaling, and chromatin remodeling. The dehydration-responsive nuclear proteome of chickpea revealed a coordinated response, which involves both the regulatory and the functional proteins.

39.4.3 Peanut

In peanut very few proteomic studies were conducted on stress response; in a recent study with selected tolerant and susceptible peanut genotypes from the US minicore collection were analyzed for changes in leaf proteins under water deficit stress [65]. A total of 102 protein bands/spots were analyzed by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) and by quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) analysis. Forty-nine nonredundant proteins were identified that implicated a variety of stress response mechanisms in peanut. It was observed that lipoxygenase and 1L-myo-inositol-1-phosphate synthase, which aid in inter- and intracellular stress signaling, were more abundant in tolerant genotypes under water deficit stress. Here, the acetyl-CoA carboxylase, a key enzyme of lipid biosynthesis, increased in relative abundance along with a corresponding increase in epicuticular wax content in the tolerant genotypes suggesting an additional mechanism for water conservation and stress tolerance. In addition, there was a marked decrease in the abundance of several photosynthetic proteins in the tolerant genotype along with a concomitant decrease in net photosynthesis in response to water deficit stress.

39.5 Metabol"omics"

Undoubtedly, transcriptomic and proteomic data are important steps in deciphering a complex biological process, but they are still insufficient since most biological processes are ultimately mediated by cell metabolites. Metabolomics is considered to provide a direct "functional readout of the physiological state" of an organism. Besides, alternative mRNA splicing, protein turnover rates, and posttranslational modifications that modulate protein activity imply that changes in the transcriptome or proteome do not always correspond to alterations in the cell metabolome [66]. Target analysis, metabolite profiling, and metabolic fingerprinting are different conceptual approaches in metabolomics that can be used for a large range of applications, including phenotyping of genetically modified plants, substantial equivalence testing, determining gene function, and monitoring responses to biotic and abiotic stresses. Metabolomics can therefore be seen as bridging the gap between genotype and phenotype. Metabolic changes underpin plant development and responses to applied stresses, and that metabolic information reflects biological endpoints more accurately than transcript or protein analysis. Hence, the only way to the complete understanding of both gene function and molecular events controlling complex plant processes is to analyze the transcriptome, the proteome, and the metabolome in an integrative manner [67].

In legumes, the metabolomic approach has been used in *M. truncatula* suspension cells to determine the responses to various stimuli [68]. Although, large-scale comprehensive metabolomic studies are difficult, a number of targeted analyses have been performed to assess the involvement of subsets of metabolites in various stresses. Although the preliminary results from combining metabolic approaches with transgenics indicates the potential of increasing intrinsic stress resistance levels in legume crops and strengthens the potential role of biotechnology in crop improvement [69, 70], it must be emphasized that most metabolic pathways are interconnected in highly complex networks. Thus, modulating one metabolic pathway may have negative impacts on another, leading to concomitant deleterious traits in the modified crop. Large-scale metabolic analyses are therefore necessary to observe the metabolic networks important for plant growth and development under a range of environmental conditions.

39.6 Gen"omics"

Genomics involves the development of molecular markers for genetic diversity analysis and it provides novel opportunities to manipulate QTL through marker-assisted selection to develop improved cultivars. The use of genetic and genomic analysis to help identify DNA regions tightly linked to agronomic traits in crops, the so-called 'molecular markers, can facilitate breeding strategies for crop improvement. The use of molecular markers for the indirect selection of improved crops can

speed up the selection process by alleviating time-consuming approaches of direct screening under greenhouse and field conditions.

39.6.1 Soybean

The availability of the soybean genome sequence in combination with the integrated genetic and physical maps are valuable resources providing soybean researchers powerful and efficient genomic tools to identify and characterize genes or QTL for agronomic traits of soybean, facilitating marker-assisted breeding and soybean improvement. In soybean, *G. max* (L.) Merr., substantial genetic variation exists for salt response. In order to identify QTL associated with salt tolerance in soybean, lines from the cross of "S-100" (salt tolerant) × "Tokyo" (salt sensitive) were evaluated in saline fields where each line was characterized with RFLP markers and an initial QTL single-factor analysis was completed. These results were used to identify genomic regions associated with the trait and to saturate the selected genomic regions with SSR markers to improve mapping precision. Subsequently, a major QTL for salt tolerance was discovered near the Sat_091 SSR marker on linkage group (LG) N. The strong relationship between the SSR marker alleles and salt tolerance suggested that these markers could be used for marker-assisted selection in commercial breeding [71] (Table 39.1).

Table 39.1 List of major identified QTL associated with abiotic stress in important legume crops.

Legume	Abiotic stress	Marker type	References
L. culinaris	Cold	RAPD	[156]
	Winter hardiness	SSR	
	Winter hardiness	SSR, RAPD AFLP	[157]
G. max	Manganese toxicity	SSR, RAPD	[158]
	Salt stress	SSR	[71]
	Waterlogging	SSR	[159]
	Phosphorus deficiency	SSR, RFLP, EST	
	Phosphorus deficiency	SSR	[160]
Medicago sativa	Aluminum toxicity	RFLP	[161]
A. hypogaea	Transpiration	SSR	[74]
	Transpiration efficiency	SSR	
	Specific leaf area (SLA)	SSR	
	SPAD chlorophyll meter reading (SCMR)	SSR	
	SPAD at stage of harvest	SSR	

SPAD: chlorophyll content; RAPD: random amplified polymorphism DNA; RFLP: restriction fragment length polymorphism; AFLP: amplified fragment length polymorphism; SSR: simple sequence repeat; EST: expressed sequence tag.

39.6.2
Chickpea

MAS is being deployed in chickpea at ICRISAT to introgress QTL alleles associated with a large root size into elite germplasm [72]. Terminal drought can curtail chickpea yield from 20% to more than 50%. Hence, a deep root system capable of extracting additional soil moisture should positively impact yield in drought-prone areas [73].

39.6.3
Peanut

At ICRISAT, the first genetic map for cultivated peanut (*A. hypogaea*), an amphidiploid (4X) species, was developed that its utility demonstrated for molecular mapping of QTL controlling drought tolerance-related traits and establishing relationships with diploid AA genome of groundnut and model legume genome species. In order to develop a genetic linkage map for tetraploid cultivated groundnut, 1145 microsatellite or simple sequence repeat (SSR) markers available in public domain as well as unpublished markers from several sources were screened on two genotypes, TAG 24 and ICGV 86031, which are parents of a recombinant inbred line mapping population. As a result, 144 (12.6%) polymorphic markers were identified that amplified 150 loci. A total of 135 SSR loci could be mapped into 22 linkage groups (LGs) [74] (Table 39.1).

39.7
Functional Genomics

Large-scale analysis by using different "omics" technologies are providing extensive data sets that will help identify potential candidate genes for an increase in intrinsic resistance and/or tolerance levels in important legume crops. Identification of these candidate genes may allow their direct application in crop improvement through MAS or genetic engineering. However, in most cases, the roles of these candidate genes remain unknown and it will be important to carry out functional studies as a preliminary step toward their use in genetic improvement. To date, the *Arabidopsis*, rice, *M. truncatula*, and *L. japonicus* genomes have been sequenced and the genome sequencing projects of some other plants is underway. The traditional pursuit of a gene starting with a phenotype (forward genetics) has paved the way for the opposite situation where the gene sequences are known but not their functions. The challenge is to decipher the function of thousands of genes identified by genome projects where reverse genetics methodologies will be the key tools. The ability to knockout genes or suppress their expression are powerful tools to determine the function of a gene. This can be done by antisense RNA suppression, targeted gene replacement, insertional mutagenesis, gene silencing through RNAi, and targeted induced local lesion in genome (TILLING) approaches.

39.7.1
Gene Silencing Approaches

Antisense RNA suppression requires considerable effort for any given target gene before even knowing whether it will be successful [75]. In *Arabidopsis*, collections of random T-DNA (over 225 000 independent *Agrobacterium* T-DNA insertions) or transposable element insertion mutants are available [76]; such a collection does not exist yet for the legumes. Targeted gene replacement via homologous recombination has not yet been reproducibly achieved for higher plants. Although collections of T-DNA mutants may be very useful, they produce a limited range of allele types and do not always produce null alleles [77, 78]. Recently, the use of the tobacco retrotransposon Tnt1 has been successfully applied for large-scale insertional mutagenesis in *M. truncatula* that promises to be a useful tool for functional genomics [79].

The term RNA silencing broadly has been adopted to describe phenomena such as posttranscriptional gene silencing (PTGS) in plants, quelling in fungi, and RNA interference in animals [80]. Researchers have developed different RNA silencing strategies as tools for selective knockout of targeted genes. Virus-induced gene silencing (VIGS) has been developed to suppress plant gene expression through infection with virus vectors that harbor a target region of the host gene [80, 81]. There are vectors available that have the ability to support VIGS in plants [82, 83]; these have not yet been used extensively in legumes.

Since VIGS in peanut is not yet feasible, 25 peanut water deficit stress-induced cDNAs were characterized in a heterologous species *Nicotiana benthamiana* [84]. Increased membrane damage was seen under water deficit stress in most of the silenced plants signifying that many of these stress-induced genes were important to confer drought tolerance. Under water stress, silencing of homologue of flavonol 3-*O*-glucosyltransferase (F3OGT), a homologue of alcohol dehydrogenase, a homologue of salt-inducible protein, and a homologue of heat shock protein 70 showed more visible wilting symptoms compared to the controls. Interestingly, downregulation of two genes, homologous to aspartic proteinase 2, and Jumonji class of transcription factor showed relative drought-tolerant phenotypes. Moreover, F3OGT-silenced plants showed more wilting symptoms, membrane damage, and chlorophyll degradation than any other type during water deficit. These results demonstrated that VIGS approach can be used to characterize and assess the functional relevance of water-deficit-stress-induced cDNAs in a heterologous species.

39.7.2
TILLING

The limitations of RNA silencing or insertational mutagenesis can be overcome by TILLING that combines chemical mutagenesis with a powerful screening method for potential mutations [75, 85, 86]. The generation of phenotypic variants without introducing foreign DNA in the plant makes TILLING very suitable not only for functional analysis but also for agricultural applications. The TILLING facility for collection of mutants is available for *L. japonicus* [87] and *M. truncatula* (U.C. Davis,

USA; CNRS, Gif-Sur-Yvette, France). TILLING facilities are also being extended to a wider variety of legumes including soybean and peanuts.

39.8 Transgenomics

The use of transgenic technology or "transgenomics" potentially offers a more targeted gene-based approach for gaining valuable information to understand the mechanisms governing stress tolerance, providing a complementary means for the genetic enhancement of field crops, thereby alleviating some of the major constraints to crop productivity in developing countries [88]. Tissue culture has been repeatedly described as difficult in grain legumes. Regeneration from both organogenesis and embryogenesis has been reported to be recalcitrant in this plant group [89, 90] and has been attributed as a major constraint in transgenic development for many legumes. Since advances in molecular genetics, for example, gene overexpression, gene suppression, promoter analysis, and T-DNA tagging require efficient transformation systems [91]. Implementation of robust protocols for regeneration in legumes is therefore a necessary condition for genetic transformation.

In plants, upon exposure to abiotic stress, a number of genes are turned on resulting in increased levels of several osmolytes and proteins that may be responsible for conferring a certain degree of protection from these stresses. Therefore, it may be necessary to transfer several potentially useful genes into the same plant in order to obtain a high degree of tolerance to drought or salt stress. Novel genes accessed from exotic sources of plants, animals, bacteria, and even viruses can be introduced into the crop through various genetic transformation methods [9] with the possibility of controlling the timing, tissue specificity, and expression level of transferred genes for their optimal function.

The feasibility of using *Agrobacterium tumefaciens*-mediated gene transfer has been an important breakthrough in legume transgenic research although the rate of recovery of transgenic lines is still low in many cases [90, 91]. To date, genetic transformation has been reported in all the major legume crops such as *Vigna* species, *C. arietinum*, *C. cajan*, *Phaseolus* spp., *Lupinus* spp., *Vicia* spp., *P. sativum*, and soybean. Despite being crucial to tropical agriculture, transgenic grain legumes with an exception of soybean have not moved out from laboratories to large farm lands compared to their counterparts, "cereals" [92]. For example, the increase in tolerance to aluminum toxicity in transgenic alfalfa [93] and cyanamide toxicity in transgenic soybean [94] demonstrates the potential of this approach in legumes (Table 39.2). At ICRISAT, efficient transformation protocols have been developed for legume crops including groundnut, pigeonpea, and chickpea. A more exhaustive review of the application of transgenesis to overcome abiotic stresses in plants is provided in Ref. [9].

Various transgenic technologies for improved stress tolerance have been developed involving the expression of functional genes including those encoding for enzymes required for the biosynthesis of osmoprotectants [95–97] or modifying membrane lipids [98, 99], late embryogenesis proteins [100], and detoxification

Table 39.2 Selective reports on production of abiotic stress-tolerant transgenic legumes.

Gene	Protein	Source(s)	Cellular role	Transhost	Promoter used	Performance of transgenics	References
NTR1 (nectarin1)	Jasmonic acid carboxyl methyltransferase	*B. campestris*	Methyl jasmonate	*G. max*	CaMV35S		[119, 120]
Cah	Cyanamide hydratase	*Myrothecium verrucaria*	Cyanamide hydratase	*G. max*	*A. thaliana* actin-2 promoter	Tolerance to cyanamide toxicity	[94]
DREB1A	DRE-binding protein	*A. thaliana*	Transcription factor	*A. hypogaea*	*A. thaliana* RD29A promoter		[112]
DREB1A	DRE-binding protein	*A. thaliana*	Transcription factor	*C. arietinum*	*A. thaliana* RD29A promoter		[Development of transgenic chickpea for drought tolerance (ICRISAT unpublished data).]
p5cs	O1-pyrroline 5-carboxylate synthase	*V. aconitifolia*	Proline biosynthesis	*C. arietinum*	CaMV35S		[122]
codA	Choline oxidase A	*Arthrobacter globiformis*	Glycine-betaine biosynthesis	*C. arietinum*	CaMV35S with a chloroplastic transit peptide		[121]
GmDREB1	DRE-binding protein	*G. max*	Transcription factor	*M. sativa*	*A. thaliana* RD29A promoter	Salt tolerance	[137]
WXP1	AP2 domain	*M. truncatula*	Wax biosynthesis	*M. sativa*	CaMV35S	Tolerance to drought	[162]
MDH	Malate dehydrogenase	*M. sativa*	Malate dehydrogenase	*M. sativa*	CaMV35S	Tolerance to aluminum toxicity	[93]

fe-sod	Fe-superoxide dismutase	*N. plumbaginifolia*	Dismutation of reactive oxygen intermediates in chloroplasts	*M. sativa*	CaMV35S with a chloroplastic transit peptide	Showed increased Fe-SOD activity, which was associated with increased winter survival	[163]
mn-sod	Mn-superoxide dismutase	*N. plumbaginifolia*	Dismutation of reactive oxygen intermediates in mitochondria	*M. sativa*	CaMV35S with a chloroplastic and mitochondrial transit peptide	Showed significantly greater survival in field under water stress and in winter	[100, 164]
sod	Superoxide dismutase	*N. plumbaginifolia*, *P. sativum*	Dismutation of toxic reactive oxygen intermediates	*M. sativa*	CaMV35S	Showed increased regrowth after freezing stress	[165]

enzymes [101]. A widely adopted strategy has been to engineer certain osmolytes for their overexpression in plants to develop stress-tolerant crops [102–107]. However, the approaches involving the transfer of a single functional gene have not proven very effective in improving plant tolerance beyond the short-term effects that have been reported [108, 109]. Hence, multiple mechanisms to engineer water stress tolerance have been utilized and studies involving regulatory genes have been more efficient [108–112].

39.8.1 Soybean

The first report of soybean (*G. max*) transformation was published in 1988 where both *Agrobacterium*-mediated transformation [113] and particle bombardment method were used [114]. At present, soybean is the only transgenic legume crop that is under commercial cultivation. Roundup ready soybean was the first transgenic soybean resistant to herbicide, commercially released in the United States in 1996 by Monsanto company (http://www.monsanto.com/history.asp), which was grown commercially in seven countries, the United States of America, Argentina, Canada, Mexico, Romania, Uruguay, and South Africa in 2001 [115]. Globally, herbicide-tolerant soybean occupied 33.3 million hectares, representing 63% of the global transgenic crop area of 52.6 million hectares for all crops by 2001 [115]. There have been numerous excellent reviews on gene technology applications in soybean [91, 116–118]. Recent reports on transgenic soybean for abiotic stress tolerance include transformation with coding sequence for cyanamide hydratase (Cah), an enzyme that converts toxic cyanamide to urea, from the soil fungus *Myrothecium*. Cah expression detoxified cyanamide in leaf callus and embryogenic cultures of soybean as well as in whole plants as shown by cyanamide resistance [94]. Another study on the constitutive expression of *nectarin1* (*ntr1*) gene from *Brassica campestris* in transgenic soybean resulted in enhanced accumulation of methyl jasmonate (MeJA). *NTR1* gene encodes jasmonic acid carboxyl methyl transferase, which is an important plant regulator involved in plant development that regulates the expression of plant defense genes in response to various stresses such as wounding, drought, and pathogens. The higher levels of MeJA in the transgenic soybean plants conferred tolerance to dehydration during seed germination and seedling growth as reflected by the percentage of the fresh weight of seedlings. In addition, the transgenic soybean plants also conferred better capacity to retain water than wild-type plants when drought tolerance was tested using detached leaves [119, 120].

39.8.2 Chickpea

Since it is believed that osmoregulation is one of the best strategies for abiotic stress tolerance, especially if osmoregulatory genes could be triggered in response to drought, salinity, and high temperature. A prokaryotic osmoregulatory choline oxidase gene (*codA*) has been targeted at the chloroplasts to enhance the potential

of photosynthetic machinery of chickpea to withstand oxidative damage. Chloroplasts from plants of transgenic lines were evaluated for their efficacy to withstand photoinhibitory damage where the loss in PS II activity in chloroplasts of wild-type plants exposed to high light intensity was significantly higher than that in chloroplasts of transgenic chickpea. The results indicated that H_2O_2 produced by *codA* as a by-product during synthesis of glycine-betaine is responsible for building stronger antioxidant system in chloroplasts of transgenic chickpea plants [121]. Similarly at ICRISAT, the P5CSF129A gene encoding the mutagenized *D1-pyrroline-5-carboxylate synthetase* (P5CS) for the overproduction of proline was introduced in chickpea. The accumulation of proline in several of these transgenic events was more pronounced and increased significantly in the leaves when exposed to water stress along with a decrease in free radicals as measured by a decrease in the malonaldehyde (MDA) levels, a lipid peroxidation product [122]. However, the overexpression of proline appeared to have no beneficial effect on biomass accumulation since only a few events showed a significant increase in the biomass production toward the end of the progressive drying period. In any case, the overexpression of P5CSF129A gene resulted only in a modest increase in the transpiration efficiency (TE), thereby indicating that the enhanced proline had little bearing on the components of yield architecture that are significant in overcoming the negative effects of drought stress in chickpea. These results agree with the previous reports in other crops [123–125]) and, in our own assessment, the gene affecting single protein might be less efficient in coping with water-limiting conditions [122].

To address the multigenicity of the plant response to stress, a strategy to target transcription factors that regulate the expression of several genes related to abiotic stress was considered. Regulatory genes or transcription factors, more specifically those belonging to the AP2/ERF family, have previously been shown to improve stress tolerance under lab conditions by regulating the coordinated expression of several stress-related genes in heterologous transgenic plants [111, 112, 126]. Hence, a large number of transgenic plants of chickpea carrying the *DREB1A* transcription factor from *Arabidopsis thalianna*, driven by a stress-inducible promoter from *rd29A* gene from *A. thaliana*, have been developed [Development of transgenic chickpea for drought tolerance (ICRISAT unpublished data).].

39.8.3 Peanut

The transfer of individual genes to plants, for acquiring higher stress tolerance, has so far had only a limited impact. However, the simultaneous transcriptional activation of a subset of those genes, by transferring transcription factors, has been revealed as a promising strategy [127, 128]. Using transgenic plants carrying regulatory genes, specifically those belonging to the AP2/EREBP family (DREB1A), proved an efficient method to improve the abiotic stress tolerance of crop plants [111, 112, 126]. The overexpression of *DREB1A* under the control of a constitutive promoter was detrimental when stress was not applied, although it had a positive effect on plants under stress. The use of the stress-inducible promoter from rd29A, instead of the

CaMV35S promoter, to overexpress *DREB1A* minimized the negative effects on plant growth [111]. Since improving the water use efficiency (WUE) of a plant is a complex issue, efforts to breed groundnut genotypes for high TE and stomatal conductance have obtained limited success. At ICRISAT, the transgenic groundnut plants carrying *DREB1A* transcription factor from *A. thaliana* driven by a stress-inducible promoter from *rd29A* gene also from *A. thaliana* have been shown to improve drought tolerance under greenhouse conditions [112]. A few transgenic events with contrasting responses have been selected for further detailed studies on the gas exchange characteristics of leaves. Besides, the biochemical responses of plants under identical conditions of water stress have been examined critically to further understand the mechanisms underlying environmental stress resistance in these transgenic events [109].

39.8.4
Candidate Genes from Legumes

There are several reports on candidate genes being cloned from legumes and tested in model plants for abiotic stress tolerance (Table 39.3). These advances suggest good prospects for developing transgenic legumes with enhanced tolerance to abiotic stress in the near future. There have been reports on manipulating the expression of pea DNA *helicase45* or the glyoxalate pathways conferring high salinity tolerance in tobacco [129, 130]. Similarly, ectopic expression of the *AhNCED1* gene (which results in oxidative cleavage of *cis*-epoxycarotenoids) in *Arabidopsis* improved the water stress tolerance levels by causing accumulation of endogenous ABA [131]. Besides, a *CarNAC1* gene (for NAM, ATAF1,2, and CUC2) was isolated from a cDNA library constructed from chickpea (*C. arietinum* L.) seedling leaves treated by polyethylene glycol and has been found to play important roles in plant development and stress responses [132]. Another cDNA clone encoding a dehydrin gene, *cpdhn1*, was isolated from a cDNA bank prepared from ripening seeds of *C. pinnatifidum* [133]. Since the gene expression was induced not only during seed development but also in leaves in response to drought, chilling, and salinity and to treatment with ABA or methyl jasmonate, the CpDHN1 protein may have a role in tolerance to a variety of environmental stresses, both abiotic and biotic. In another effort, a *CAP2* gene from chickpea encoding a novel AP2 family transcription factor that increased under dehydration has been characterized [134]. The CaMV35S promoter-driven expression of CAP2 in tobacco resulted in increased tolerance to dehydration and salt stress than the wild-type plants. Besides, transgenic plants expressed higher steady-state transcript levels of abiotic stress response genes NtERD10B and NtERD10C and auxin response genes IAA4.2 and IAA2.5, indicating a mutual interrelation between plant growth and development and abiotic stress response pathways and a probable involvement of CAP2 in both the signaling pathways.

Several transcription factors of AP2 family including DREB homologue and ERF transcription factors have been isolated from soybean and were characterized by their expression in transgenic plants. GmDREB2 [135] GmDREB3 [136] from soybean was expressed in *Arabidopsis* and has shown tolerance to drought and salt stress, whereas

Table 39.3 Abiotic stress-responsive genes characterized from legumes.

Gene	Protein	Source(s)	Cellular role	Transhost	Promoter used	Tolerance	Reference
CAP2	AP2/EREBP	*C. arietinum*	Transcription factor	*N. tabacum*	CaMV35S	Drought and salt tolerance	[134]
bip	Binding protein	*G. max*	Molecular chaperone involved in unfolded protein response	*N. tabacum*	CaMV35S	Water stress	[166]
GmbZIP44, GmbZIP62, and GmbZIP78	bZIP	*G. max*	Transcription factor	*Arabidopsis*	CaMV35S	Salt and freezing stresses	[141]
GmCHI	Chilling inducible	*G. max*	Transcription factor	*Arabidopsis*	CaMV35S	Cold, drought, and salt tolerance	[143]
GmDREB2	AP2/EREBP	*G. max*	Transcription factor	*Arabidopsis*	CaMV35S	Drought and salt tolerance	[135]
GmDREB3	AP2/EREBP	*G. max*	Transcription factor	*Arabidopsis* and *N. tabacum*	CaMV35S *A. thaliana* RD29A promoter		[136]
GmDREBa, GmDREBb, and GmDREBc	AP2/EREBP	*G. max*	Transcription factor	Yeast one hybrid	—	Response to abiotic stresses	[138]
GmERF057 and GmERF089	AP2/EREBP	*G. max*	Transcription factor	*N. tabacum*	CaMV35S promoter	Drought and salt	[139]
GmGT-2A and GmGT-2B	Trihelix	*G. max*	Transcription factor	*Arabidopsis*	—	Abiotic stresses	[144]
GmMYB76, GmMYB177, and GmMYB92	MYB	*G. max*	Transcription factor	*Arabidopsis*	CaMV35S	Salt and freezing	[140]
GmPHD2	Alfin1-type PHD finger protein	*G. max*	Transcription factor	*Arabidopsis*	CaMV35S	Salt tolerance	[145]
GmTP55, antiquitin homologue	ALDH7 family	*G. max*	Abiotic stress responsive	*N. tabacum* and *Arabidopsis*	CaMV35S	Drought and salt	[146]

(*Continued*)

Table 39.3 (*Continued*)

Gene	Protein	Source(s)	Cellular role	Transhost	Promoter used	Tolerance	Reference
GmWRKY13	WRKY	*G. max*	Transcription factor	*Arabidopsis*	CaMV35S	Increased sensitivity to salt and mannitol stress	[142]
GmWRKY21	WRKY	*G. max*	Transcription factor	*Arabidopsis*	CaMV35S	Cold stress	[142]
GmWRKY54	WRKY	*G. max*	Transcription factor	*Arabidopsis*	CaMV35S	Salt and drought	[142]
SCOF-1 soybean cold-inducible factor-1	C2H2 (Zn)	*G. max*	Transcription factor	*Arabidopsis* and *N. tabacum*	CaMV35S	Cold	[167]
alfin1	Zn finger family of proteins	*M. sativa*	Transcription factor	*M. sativa*	CaMV35S	Salt	[168]
msalr	NADPH-dependent aldose/aldehyde	*M. Sativa*	Detoxification	*N. tabacum*	CaMV35S	Improved recovery after rehydration	[169]
Mszpt2-1	Kruppel like	*M. truncatula*	Transcription factor	*M. truncatula*	CaMV35S	Gene-silenced transgenics became more sensitive to recover from salt stress	[170]
WXP1, WXP2	AP2 domain	*M. truncatula*	Wax biosynthesis	*Arabidopsis*	CaMV35S	Drought	[171]
Ph_acut_ AY026054	bZIP	*Phaseolus acutifolius*	Transcription factor	—	—	Water deficit stress	[172]
Ph_vulg_AF350505	bZIP	*P. vulgaris*	Transcription factor	—	—	Water deficit stress	[172]
PvNAP	NAC	*P. vulgaris*	Transcription factor	atnap null mutant, *Arabidopsis*	AtNAP	Leaf senescence	[173]

GmDREB1 enhanced salt tolerance in transgenic *medicago* [137]. Besides, three more DREB homologues were identified in soybean, namely, GmDREBa, GmDREBb, and GmDREBc. While *GmDREBa* and *GmDREBb* genes were induced by salt, drought, and cold stresses in the leaves of soybean seedlings, the expression of *GmDREBc* was apparently induced in roots by salt, drought, and abscisic acid treatments [138]. In another study, expression analysis of ERF transcription factors in soybean showed that nine unigenes belonging to six ERF family subgroups were induced by both biotic/abiotic stresses and hormone treatment, suggesting that they were involved in crosstalk between biotic and abiotic stress-responsive signaling pathways. Overexpression of two full-length soybean genes GmERF057 and GmERF089 from two different subgroups enhanced the tolerances to drought, salt stresses, and/or pathogen infection of the tobacco plants [139]. Moreover, transcription factors of MYB.family GmMYB76, GmMYB177, and GmMYB92 [140] and of bZIP family GmbZIP44, GmbZIP62, and GmbZIP78 were isolated from soybean and tested in transgenic *Arabidopsis* for their role in stress tolerance [141]. Over 64 *GmWRKY*genes from soybean were identified that expressed differentially under various abiotic stresses. For example, GmWRKY21 responded to cold stress, while GmWRKY54 conferred salt and drought tolerance, possibly through the regulation of DREB2A and STZ/Zat10 [142]. Also, six GmPHD genes encoding Alfin1-type PHD finger proteins were identified in soybean and their expressions responded differentially to drought, salt, cold, and ABA treatments. Another gene GmCHI (chilling inducible) has been assumed to be regulated by ABA-dependent signal transduction pathway during cold acclimation in soybean. Overexpression of *GMCHI* in *Arabidopsis* under the control of CaMV35S promoter enhanced the tolerance to cold, drought, and NaCl stresses [143]. In another report, GmGT-2A and GmGT-2B, "GT" element binding transcription factors belonging to the trihelix family genes, were cloned from soybean and their overexpression improved plant tolerance to salt, freezing, and drought stress in transgenic *Arabidopsis* plants [144]. Transgenic *Arabidopsis* plants overexpressing the GmPHD2 showed salt tolerance compared to the wild-type plants by diminishing the oxidative stress through regulation of downstream genes [145]. Similarly, an ectopic expression of a soybean antiquitin homologue gene GmTP55 (closely related to the stress-induced plant antiquitin-like proteins belonging to the ALDH7 family) in both *Arabidopsis* and tobacco has been shown to confer tolerance to salinity during germination and to water deficit during plant growth [146].

39.9 Phen"omics"

Although occupying the last position in a long and wide array of gene-based "omics" approaches, phenomics, which can be viewed as a "modern phenotyping counterpart," is critical to the gene-"omics" approach. Indeed, it is often and wisely considered that unless the phenotypic expression of plants displaying different genomic/metabolomic/proteomic/transcriptomic/transgenomic content is properly understood and characterized, and then accurately and precisely measured, there is

little chance that any of the approaches above can be successful. There is unfortunately an increasing gap between the knowledge on the genotype and that on the phenotype [147] that urgently needs to be tackled. What is often viewed in the phenomics is the possibility to harness new technology to increase the throughput of "traditional" phenotypic assessments. While this has indeed a tremendous potential, it also bears the risk of making phenotyping a technology-driven activity generating (many) numbers, rather than a question/hypothesis-driven approach to the understanding of plant response to stress. In what follows, we attempt to lay out the basic principles that should be considered when attempting "phenomics" characterization for focusing on the type of abiotic stress (e.g., drought, salinity, etc.).

39.9.1 Relevant Protocols to Assess Plant Response to Stress: Drought as a Case

There have been a number of studies that explain the importance of using relevant protocols to assess drought stress response [108] or in the approach to look at specific traits that are likely to be beneficial under water limitation, like root systems [148]. In short, the principle of exposing plants to stress is about ensuring that the kinetics of stress impositions are relevant to those that plants would face in natural environments. The use of rapid stress imposition (uprooting, exposure to PEG, growth in very small pots, etc.) is not suitable to properly characterize plant response to stress, and especially to acquire knowledge on the genes involved in the plant response, as these are likely to be different from the genes that would be expressed under natural conditions. Therefore, while applying water stress, it is essential to have a rigorous control and record of the stress intensity and the kinetics of stress imposition. One school of thought proposes to look at stress intensity from the angle of the soil moisture available for transpiration [149], as it has the great and powerful advantage of allowing comparison across all plant species, across environments. Unfortunately, rarely care is taken for this index in many gene-based studies. The other school of thought is to measure leaf water potential as an indicator for stress intensity. It has the drawback of being more labor intensive and less sensible than simple gravimetrics of soil moisture measurement [150, 151], but has the value of providing information on leaf water status that can be useful for understanding the other "omics" responses. In any case, any of these two "stress indicators" is a key requirement to make any sense of "omics" responses to water deficit. Equally important is the need to measure the environmental conditions under which plants are assessed. Much of the gene-based "omic" work takes place in glasshouse or growth chamber environment, where it is essential to assess air temperature, humidity, and light intensity to understand the physical drivers of plant water use.

39.9.2 Relevant Protocols Used to Extract "Omics" Products in Grain Legumes

In recent past, a large number of studies have attempted to identify genes responsible for stress response. Besides the fact that there are often thousands of genes that are

expressed, making the choice of key ones, if any, the conditions under which the plants are challenged to stress are often questionable. One such example is a recent study [40] that reports 319 unique ESTs from two contrasting lines of chickpea, with 70% of these being more than twofold abundant in the tolerant cultivar. The protocol used to challenge the plant was withdrawal of irrigation at 12 days after sowing, for a period of 3, 6, and 12 days. Here, the plants were grown in pots (3L) containing a composite soil, without any indication of the soil water capacity. Besides, no measurement of soil moisture was done and only relative water content was measured, putatively as a control for moisture stress. In another study on chickpea [45], attempts were made to expose plants to stress conditions that were similar to those of the natural conditions. Here, a dry-down technique was used to expose the plants to a progressive water stress, similar to the one in the field conditions, by partially compensating the daily water loss and ensuring that water stress symptoms (apparent from a decrease in plant transpiration) do not occur until at least 10 days after stress imposition, that is, similar to the field conditions. In such experiments, the soil moisture, which indicates the level of stress, is kept rigorously constant across genotypes tested. It allows replication of the experiment across environments or plant materials. Moreover, the contrasting materials were also challenged for salinity tolerance. The protocol used here was the very same protocol as used to screen genotypes for seed yield under salt stress. Since the physiological analysis also indicates that reproduction is likely the most sensitive process under salt stress, the flower tissue samples collected during the study for genotyping.

Similarly, work has been carried out in peanut to identify ESTs involved in the contrasting drought response in two genotypes (TAG24 and ICGV86031) (unpublished – EST sequence posted in Genbank). While TAG24 appears to have a high threshold of soil moisture where its transpiration declines, ICGV86031 clearly declines transpiration at lower soil moisture (dryer soil). Such differences are expected to play a causal role in the transpiration efficiency differences between these two lines. To identify possible genes responsible for that response, a standard dry-down protocol was used [153, 154], where tissue sampling was performed precisely when genotypes displayed phenotypic differences (differences in the transpiration relative to the control) during the stress. These two examples illustrate that relevant protocols are needed to mimic as closely as possible the natural conditions, to extract genes that are most likely to be involved in the response under natural conditions.

39.9.3
Adaptive versus Constitutive Genes

In most of the cases, the gene-based "omic" approaches tend to be influenced a lot by the idea that stress tolerance "results" from different stress-responsive genes intervening in the case of tolerant entries and being absent/unexpressed in sensitive lines. However, as far as water limitation is concerned, plants exposed to water deficit usually behave like fully irrigated plants until about 60% or more of the soil moisture has been depleted [152]. So, understanding how plants control plant water use before

stress symptoms appear is even more important than understanding how plants respond when they are left with only 40% or less of the soil water. A recent study on pearl millet shows this is critical for the terminal drought tolerance [153, 154]. Here, differences in leaf conductance under fully irrigated conditions were identified and related to the yield-based differences under stress. So, this means that genotypes have an array of development and functioning characteristics displayed under nonstressed conditions that can determine how well they would be adapted to a situation of stress. In the example of pearl millet cited above, a lower leaf conductance under fully irrigated conditions would simply limit water use when water is available and make it available for the grain filling period, a time when soil moisture has receded and plants are under stress. Therefore, constitutive traits become critical to consider in the "omic" approaches, including phenomics, to first identify their mechanisms (e.g., a slower leaf expansion rate or smaller leaf size) and then the related genes involved in development or functioning processes (e.g., a limited leaf conductance) that predispose particular genotypes to be better equipped to face a forthcoming water limitation.

39.9.4 Physiology Integration in a Novel Context of Environment-Specific Breeding

The growing genotype–phenotype gap is in part explained by a generational change in plant biologists, who have turned away from disciplines of physiology–biochemistry to molecular genetics, and by the belief that a single gene approach of "tolerance gene" identification would solve all problems. Rather, there is a clear need to have the phenotypic information guiding the gene-based "omics" work. Hence, phenomics should in part include a reductionist approach to break down integrated measurement of traits such as yield or biomass into smaller, more heritable components or traits, closer to the identification of cell- or organ-based mechanisms responsible for the integrated response differences. Again, molecular "omics" offer the potential for easier and more reliable way of predicting phenotypes with the condition that robust phenotype–genotype relationships have first been demonstrated. In any case, a reductionist approach to understand the mechanisms of tolerance to abiotic stress is needed to progress toward the identification of genes involved. It also fits the likely evolution of breeding approach from a one-variety-to-fit-all-situation to environment-specific breeding where it will be critical to understand/identify particular characteristics making a genotype adapted to particular environments.

Physiology as a discipline is an integral component of such a breeding perspective. The approaches and protocols that are developed by "phenomists" need to be adapted, or adaptable, to the requirement of a breeding program: these need to be large scale, simple, and applicable to a large number of entries, which is a prerequisite for QTL mapping, either through RIL population or through association panels. At the same time, these need to be capable of assessing cell- or organ-based mechanisms having potential importance. For instance, recent work in pearl millet indicates that lower leaf conductance leads to having water left in the soil profile to support seed filling, and this is attributed to differences in root hydraulics [154], for which precise

protocols are needed. This is a prerequisite to identify the genes involved in a cell- or organ-based mechanism.

39.9.5 Addressing Complexity of Plant Response to Abiotic Stress

Phenomics is also about addressing the complexity of plant response to stress. For instance, crop success under terminal drought could be explained by genotype's capacity to extract water deeper from the soil profile and make this water available for critical periods. In parallel, having water available for critical periods could be explained by differences in the pattern of water use (less water use) before reaching such critical development stages. The later could lead to less water use, while the former could lead to earlier/more water use. So, while this small example illustrates the need to target specific mechanisms, it also stresses on the need to look at different traits in a comprehensive manner. The difficulty lies in having an experimental approach that is enough reductionist to accurately phenotype cell- or organ-based actions, while being sufficiently integrated to have such reductionist measurements coupled to "integrated" measurements that have a meaning for the breeding community. At ICRISAT, work is ongoing where the initial target is to unravel the functionality of rooting traits in a way that their actual combination with terminal water deficit can be understood [148, 155]. As the work progresses, the initial focus on roots, root functionality, and water capture is getting complemented by a component of understanding of the regulation of water use by the crop canopy. Hence, modeling is surely a critical component of the breeding program, to reintegrate the pieces of the phenomics puzzle in a comprehensive and relevant framework. With the present phenomics development, allowing for measuring more and more, modeling remains a sort of safeguard that helps target what phenotype matters more than those that matter less. At the same time, the combination of phenomics and modeling offers a great potential of rapidly assessing the value of certain phenotypes on plant performance.

39.10 Conclusions

Over the years, biotechnology has emerged as a promising tool to overcome stresses in plants; but to date progress has been limited in legumes. Biotechnological applications, including all "omics," were direct and potential approaches for improving abiotic stress tolerance in grain legumes where the existing germplasm lacks the required traits for conventional breeding. However, successful application of "omics" to abiotic constraints requires knowledge of stress response at molecular level, which includes gene expression to protein or metabolite and its phenotypic effects. Availability of genome sequence of model legumes and soybean has a potential to facilitate positional cloning and other approaches and their applications for abiotic stress research on legumes. A genome-wide expression profiling with next-gener-

ation sequencing approaches could circumvent the problems posed by extremely large genomes like legumes.

Compared to analysis of the transcriptome, analysis of the plant proteome and metabolome in response to abiotic stresses is still limited to *M. truncatula* and protein reference maps of soybean to stress responses are now available. More recently, there are few proteomics studies on peanut and chickpea available, and they have to be extensively carried out in all grain legumes for abiotic stress tolerance. Moreover, the recent progress in the mass-scale profiling of the genome, transcriptome, proteome, and metabolome (i.e., "omics") offers the possibility of investigating the concerted response of thousands of genes to drought and other abiotic stresses. Hence, the research dealing with other strategies such as MAS or even classical breeding will be able to take advantage of the results being gathered from these "omics" technologies.

The mapping of abiotic stress QTL in legume is still at an early stage and gene pyramiding has not been applied yet. Nevertheless, with the establishment of the model legumes, *M. truncatula* and *L. japonicus*, there is now applicable information on legumes. Among the grain legumes, soybean has been more intensively studied, and the availability of more numbers of ESTs and genome sequences will facilitate mapping of major QTL in other legumes. The use of transgenic technology potentially offers a more targeted gene-based approach not only for gaining valuable information but also improving stress tolerance in legumes. However, the genetic engineering options addressing plant resistance to abiotic stress, mainly in relation to drought, have been confined to experimental laboratory work and to single gene approaches, lead to marginal stress improvement in grain legumes. Hence, there is a need for identification of candidate genes for abiotic stress tolerance in legumes that will allow their direct application in genetic engineering. Hence, multiple mechanisms to engineer abiotic stress tolerance and studies involving regulatory genes under the control of stress-inducible promoters have a potential to improve stress tolerance in grain legumes. Also, since only transgenic soybean has been commercialized in developed countries, there is a need to address the regulatory issues for transgenics' deployment in developing countries. Needless to point out that the current advances in tissue-derived techniques, genetic transformation and MAS, together with the advances in powerful new "omics" technologies offer a great potential to improve this situation. Besides, a thorough and meaningful assessment of phenotypic expression to understand the mechanisms of adaptation to stress is needed before genes responsible for these mechanisms can be identified and tagged. Indeed, it is now possible to target almost all legume crops with a variety of biotechnological approaches for genetic improvement.

References

1 Graham, P.H. and Vance, C.P. (2003) Legumes: importance and constraints to greater use. *Plant Physiol.*, **131**, 872–877.

2 Popelka, J.C., Terryn, N., and Higgins, T.J.V. (2004) Gene technology for grain legumes: can it contribute to the food challenge in developing countries? *Plant Sci.*, **167**, 195–206.

3 Vance, C.P., Graham, P.H., and Allan, D.L. (2000) Biological nitrogen fixation

phosphorus: a critical future need, in *Nitrogen Fixation: from Molecules to Crop Productivity* (eds F.O. Pedrosa, M., Hungria, M.G., Yates, and W.E. Newton), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 506–514.

4 National Academy of Science (1994) *Biological Nitrogen Fixation*, National Academy Press, Washington, DC.

5 Boyer, J.S. (1982) Plant productivity and environment. *Science*, **218**, 443–448.

6 Bray, E.A., Bailey-Serres, J., and Weretilnyk, E. (2000) Responses to abiotic stresses, in *Biochemistry and Molecular Biology of Plants* (eds W. Gruissem, B. Buchannan, and R. Jones), American Society of Plant Physiologists, Rockville, MD, pp. 1158–1249.

7 Dita, M.A., Rispail, N., Prats, E., Rubiales, D., and Singh, K.B. (2006) Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. *Euphytica*, **147**, 1–24.

8 Reddy, A.R., Chaitanya, K.V., and Vivekanandan, M. (2004) Drought induced responses of photosynthesis and antioxidant metabolism in higher plants. *J. Plant Physiol.*, **161**, 1189–1202.

9 Sharma, K.K. and Lavanya, M. (2002) Recent developments in transgenics for abiotic stress in legumes of the semi-arid tropics. JIRCAS Working Report, 61–73.

10 Postel, S.L. (2000) Entering an era of water scarcity. *Ecol. Appl.*, **10**, 941–948.

11 Arrus, K., Blank, G., Abramson, D., Clear, R., and Holley, R.A. (2005) Aflatoxin production by *Aspergillus flavus* in Brazil nuts. *J. Stored Prod. Res.*, **41**, 513–527.

12 Mahmoud, A.L.E. and Abdalla, M.H. (1994) Natural occurrence of mycotoxins in broad bean (*Vicia faba* L) seeds and their effect on *Rhizobium*–Legume symbiosis. *Soil Biol. Biochem.*, **26**, 1081–1085.

13 Van Hoorn, J.W., Katerji, N., Hamdy, A., and Mastrorilli, M. (2001) Effect of salinity on yield and nitrogen uptake of four grain legumes and on biological nitrogen contribution from the soil. *Agric. Water Manage.*, **51**, 87–98.

14 Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.*, **2**, 48–54.

15 Bray, E.A. (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. *J. Exp. Bot.*, **55**, 2331–2341.

16 Anderson, J.P., Thatcher, L.F., and Singh, K.B. (2005) Plant defence responses: conservation between models and crops. *Funct. Plant Biol.*, **32**, 21–34.

17 Cook, D.R. (1999) *Medicago truncatula:* a model in the making! Commentary. *Curr. Opin. Plant Biol.*, **2**, 301–304.

18 Handberg, K. and Stougaard, J. (1992) *Lotus japonicus*: an autogamous, diploid legume species for classical and molecular genetics. *Plant J.*, **2**, 487–496.

19 Kato, T., Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E., and Tabata, S. (2003) Structural analysis of a *Lotus japonicus* genome V sequence features and mapping of sixty-four TAC clones which cover the 64 Mb regions of the genome. *DNA Res.*, **10**, 277–285.

20 Asamizu, E., Nakamura, Y., Sato, S., and Tabata, S. (2004) Characteristics of the *Lotus japonicus* gene repertoire deduced from large-scale expressed sequence tag (EST) analysis. *Plant Mol. Biol.*, **54**, 405–414.

21 Kulikova, O., Gualtieri, G., Geurts, R., Kim, D.J., Cook, D., Huguet, T., de Jong, J.H., Fransz, P.F., and Bisseling, T. (2001) Integration of the FISH pachytene and genetic maps of *Medicago truncatula*. *Plant J.*, **27**, 49–58.

22 Pedrosa, A., Sandal, N., Stougaard, J., Schweizer, D., and Bachmair, A. (2002) Chromosomal map of the model legume *Lotus japonicus*. *Genetics*, **161**, 1661–1672.

23 Thoquet, P., Gherardi, M., Journet, E.P., Kereszt, A., Ane, J.M., Prosperi, J.M., and Huguet, T. (2002) The molecular genetic linkage map of the model legume *Medicago truncatula*: an essential tool for comparative legume genomics and the isolation of agronomically important genes. *BMC Plant Biol.*, **2**, 1.

24 Kalo, P., Seres, A., Taylor, S.A., Jakab, J., Kevei, Z., Kereszt, A., Endre, G., Ellis, T.H.N., and Kiss, G.B. (2004) Comparative mapping between *Medicago sativa* and *Pisum sativum*. *Mol. Genet. Genomics*, **272**, 235–246.

25 Stracke, S., Sato, S., Sandal, N., Koyama, M., Kaneko, T., Tabata, S., and Parniske, M. (2004) Exploitation of colinear relationships between the genomes of *Lotus japonicus*, *Pisum sativum* and *Arabidopsis thaliana*, for positional cloning of a legume symbiosis gene. *Theor. Appl. Gen.*, **108**, 442–449.

26 Lievens, S., Goormachtig, S., and Holster, M. (2001) A critical evaluation of differential display as a tool to identify genes involved in legume nodulation: looking back and looking forward. *Nucleic Acids Res.*, **29**, 3459–3468.

27 Wan, J.S., Sharp, J.S., Poirier, G.M.C., Wagaman, P.C., Chambers, J., Jayashree, P., Horn, Y.-L., Galindo, J.E., Huvar, A., Peterson, P.A., Jackson, M.R., and Erlande, M.G. (1996) Cloning differentially expressed mRNAs. *Nat. Biotechnol*, **14**, 1685–1691.

28 Ramanjulu, S. and Bartels, D. (2002) Drought- and desiccation-induced modulation of gene expression in plants. *Plant Cell Environ.*, **25**, 141–151.

29 Ishitani, M., Rao, I., Wenzl, P., Beebe, S., and Tohme, J. (2004) Integration of genomics approach with traditional breeding towards improving abiotic stress adaptation: drought and aluminum toxicity as case studies. *Field Crop Res.*, **90**, 35–45.

30 Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T. *et al.* (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold, and high-salinity stresses using a full-length cDNA microarray. *Plant J.*, **31**, 279–292.

31 Crespi, M. (2007) Abiotic stress in legumes: analysis of the response to abiotic stress in legumes. Available at http://www.grainlegumes.com.

32 Singh, B.N., Mishra, R.N., Agarwal, P.K., Goswami, M., Nair, S., Sopory, S.K., and Reddy, M.K. (2004) A pea chloroplast translation elongation factor that is regulated by abiotic factors. *Biochem. Biophys. Res. Commun.*, **320**, 523–530.

33 Thompson, R., Ratet, P., and Kuster, H. (2005) Identification of gene functions by applying TILLING and insertional mutagenesis strategies on microarray-based expression data. *Grain Legumes*, **41**, 20–22.

34 Oldroyd, G. (2005) Sequencing the model legume *Medicago truncatula*. *Grain Legumes*, **41**, 23.

35 Lamblin, A.-F.J., Crow, J.A., Johnson, J.E. *et al.* (2003) MtDB: a database for personalized data mining of the model legume *Medicago truncatula* transcriptome. *Nucleic Acids Res.*, **31**, 196–201.

36 Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W. *et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature*, **437**, 376–380.

37 Ohtsu, K., Smith, M.B., Emrich, S.J., Borsuk, L.A., Zhou, R., Chen, T., Zhang, X., Timmermans, M.C.P., Beck, J., Buckner, B., Buckner, D.J., Nettleton, D., Scanlon, M.J., and Schnable, P.S. (2007) Global gene expression analysis of the shoot apical meristem of maize (*Zea mays* L). *Plant J.*, **52**, 391–404.

38 Umezawa, T., Mizuno, K., and Fujimura, T. (2002) Discrimination of genes expressed in response to the ionic or osmotic effect of salt stress in soybean with cDNA-AFLP. *Plant Cell Environ.*, **25**, 1617–1625.

39 Medini, M., Baum, M., and Hamza, S. (2009) Transcript accumulation of putative drought responsive genes in drought-stressed chickpea seedlings. *Afr. J. Biotechnol.*, **8**, 4441–4449.

40 Jain, D. and Chattopadhyay, D. (2010) Analysis of gene expression in response to water deficit of chickpea (*Cicer arietinum* L.) varieties differing in drought tolerance. *BMC Plant Biol.*, **10**, 24.

41 Mantri, N.L., Ford, R., Coram, T.E., and Pang, E.C.K. (2007) Transcriptional profiling of chickpea genes differentially regulated in response to high-salinity, cold and drought. *BMC Genomics*, **8**, 303.

42 Gao, W.R., Wang, X.S., Liu, Q.Y., Peng, H., Chen, C., Li, J.G., Zhang, J.S., Hu, S.N., and Ma, H. (2008) Comparative analysis of ESTs in response to drought stress in chickpea (*C. arietinum* L). *Biochem. Biophys. Res. Commun.*, **376**, 578–583.

43 Jayashree, B., Buhariwalla, H.K., Shinde, S., and Crouch, J.H. (2005) A legume genomics resource: the Chickpea Root Expressed Sequence Tag Database. *Electron. J. Biotechnol. [Online]*, **8** (2). Available at http://www.ejbiotechnology.info/content/vol2.

44 Buhariwalla, H.K., Jayashree, B., Eshwar, K., and Crouch, J.H. (2005) Development of ESTs from chickpea roots and their use in diversity analysis of the *Cicer* genus. *BMC Plant Biol.*, **5**, 16.

45 Varshney, R.K., Hiremath, P.J., Lekha, P., Kashiwagi, J., Balaji, J., Deokar, A.A., Vadez, V., Xiao, Y., Srinivasan, R., Gaur, P.M., Siddique, K.H.M., Town, C.D., and Hoisington, D.A. (2009) A comprehensive resource of drought- and salinity-responsive ESTs for gene discovery and marker development in chickpea (*Cicer arietinum* L). *BMC Genomics*, **10**, 523.

46 Molina, C., Rotter, B., Horres, R., Udupa, S.M., Besser, B., Bellarmino, L., Baum, M., Matsumura, H., Terauchi, R., Kahl, G., and Winter, P. (2008) SuperSAGE: the drought stress-responsive transcriptome of chickpea roots. *BMC Genomics*, **9**, 553.

47 Jain, A.K., Basha, S.M., and Holbrook, C.C. (2001) Identification of drought-responsive transcripts in peanut (*Arachis hypogaea* L). *Eur. J. Biochem.*, **4**, 59–67.

48 Guo, B.Z., Yu, J., Holbrook, C.C., Lee, R.D., and Lynch, R.E. (2003) Application of differential display RT-PCR and EST/microarray technologies to the analysis of gene expression in response to drought stress and elimination of aflatoxin contamination in corn and peanut. *Toxin Rev.*, **22**, 287–312.

49 Srinivas Reddy, D. (2008) Identification and isolation of putative disease resistance gene homologues from groundnut and studies on regulatory gene expression in transgenic groundnut under abiotic stress. Ph.D. Thesis, Jawaharlal Nehru Technological University Hyderabad (JNTUH), Hyderabad, 500 085, India.

50 Guo, B., Chen, X., Dang, P., Scully, B.T., Liang, X., Holbrook, C.C., Yu, J., and Culbreath, A.K. (2008) Peanut gene expression profiling in developing seeds at different reproduction stages during *Aspergillus parasiticus* infection. *BMC Dev. Biol.*, **8**, 12.

51 Luo, M., Dang, P., Guo, B.Z., He, G., Holbrook, C.C., Bausher, M.G., and Lee, R.D. (2005) Generation of expressed sequence tags (ESTs) for gene discovery and marker development in cultivated peanut. *Crop Sci.*, **45**, 346–353.

52 Proite, K., Leal-Bertioli, S.C.M., Bertioli, D.J., Moretzsohn, M.C., da Silva, F.R., Martins, N.F., and Guimaraes, P.M. (2007) ESTs from a wild *Arachis* species for gene discovery and marker development. *BMC Plant Biol.*, **7**, 7.

53 Govind, G., Harshavardhan, V., Thamme Gowda, P., Jayaker, K., Dhanalakshmi Ramchandra, I., Senthil Kumar, M., Sreenivasulu, N., and Udaya Kumar, M. (2009) Identification and functional validation of a unique set of drought induced genes preferentially expressed in response to gradual water stress in peanut. *Mol. Genet. Genomics*, **281**, 591–605.

54 Payton, P., Kottapalli, K.R., Rowland, D., Faircloth, W., Guo, B., Burow, M., Puppala, N., and Gallo, M. (2009) Gene expression profiling in peanut using high density oligonucleotide microarrays. *BMC Genomics*, **10**, 265.

55 Gygi, S.P., Rochon, Y., Franza, B.R., and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Mol. Cell Biol.*, **19**, 1720–1730.

56 Jorrin, J.V., Rubiales, D., Dumas-Gaudot, E., Recorbet, G., Maldonado, A., Castillejo, M.A., and Curto, M. (2006) Proteomics: a promising approach to study biotic interaction in legumes: a review. *Euphytica*, **147**, 37–47.

57 Canovas, F., Dumas-Gaudot, E., Recorbet, G., Jorrin, J., Mock, H.-P., and Rossignol, M. (2004) Plant proteome analysis. *Proteomics*, **4**, 285–298.

58 Kim, S.T., Cho, K.S., Yu, S., Kim, S.G., Hong, J.C., Han, C.-D., Bae, D.W., Nam, M.H., and Kang, K.Y. (2003) Proteomic analysis of differentially expressed proteins induced by rice blast fungus and elicitor in suspension-cultured rice cells. *Proteomics*, **3**, 2368–2378.

59 Kav, N.N.V., Srivastava, S., Goonewardene, L., and Blade, S.F. (2004) Proteome-level changes in the roots of *Pisum sativum* in response to salinity. *Ann. Appl. Biol.*, **145**, 217–230.

60 Repetto, O., Bestel-Corre, G., Dumas-Gaudot, E., Berta, G., Gianinazzi-Pearson, V., and Gianinazzi, S. (2003) Targeted proteomics to identify cadmium-induced protein modifications in *Glomus mosseae*-inoculated pea roots. *New Phytol.*, **157**, 555–567.

61 Lei, Z., Elmer, A.M., Watson, B.S., Dixon, R.A., Mendes, P.J., and Sumner, L.W. (2005) A two-dimensional electrophoresis proteomic reference map and systematic identification of 1367 protein from a cell suspension culture of the model legume *Medicago truncatula*. *Mol. Cell. Proteomics*, **4**, 1812–1825

62 Sakata, K., Ohyanagi, H., Nobori, H., Nakamura, T., Hashiguchi, A., Nanjo, Y., Mikami, Y., Yunokawa, H., and Komatsu, S. (2009) Soybean Proteome Database: a data resource for plant differential omics. *J. Proteome Res.*, **8**, 3539–3548.

63 Bhushan, D., Pandey, A., Choudhary, M., Datta, A., Chakraborty, S., and Chakraborty, N. (2007) Comparative proteomics analysis of differentially expressed proteins in chickpea extracellular matrix during dehydration stress. *Mol. Cell. Proteomics*, **6**, 1868–1884.

64 Pandey, A., Chakraborty, S., Datta, A., and Chakraborty, N. (2008) Proteomics approach to identify dehydration responsive nuclear proteins from chickpea (*Cicer arietinum* L). *Mole. Cell. Proteomics*, **7**, 88–107.

65 Kottapalli, K.R., Rakwal, R., Shibato, J., Burow, G., Tissue, D., Burke, J., Puppala, N., Burow, M., and Payton, P. (2009) Physiology and proteomics of the water-deficit stress response in three contrasting peanut genotypes. *Plant Cell Envi.*, **32**, 380–407.

66 Sumner, L.W., Mendes, P., and Dixon, R.A. (2003) Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry*, **62**, 817–836.

67 Dixon, R.A. (2001) Natural products and plant disease resistance. *Nature*, **411**, 843–847.

68 Bell, C.J., Dixon, R.A., Farmer, A.D., Flores, R., Inman, J., Gonzales, R.A., Harrison, M.J., Paiva, N.L., Scott, A.D., Weller, J.W., and May, G.D. (2001) The *Medicago* Genome Initiative: a model legume database. *Nucleic Acids Res.*, **29**, 114–117.

69 He, X.Z. and Dixon, R.A. (2000) Genetic manipulation of isoflavone 7-*O*-methyltransferase enhances biosynthesis of 4′-*O*-methylated isoflavonoid phytoalexins and disease resistance in alfalfa. *Plant Cell*, **12**, 1689–1702.

70 Wu, Q.D. and Van Etten, H.D. (2004) Introduction of plant and fungal genes into pea (*Pisum sativum* L) hairy roots reduces their ability to produce pisatin and affects their response to a fungal pathogen. *Mol. Plant–Microbe. Interact.*, **17**, 798–804.

71 Lee, G.J., Boerma, H.R., Villagarcia, M.R., Zhou, X., Carter, T.E., Jr., Li, Z., and Gibbs, M.O. (2004) A major QTL conditioning salt tolerance in S-100 soybean and descendent cultivars. *Theor. Appl. Genet.*, **109**, 1610–1619.

72 Saxena, N.P., Krishanamurthy, L., and Johansen, C. (2002) Genetic improvement of drought in chickpea at ICRISAT, in *Field Screening for Drought Tolerance in Crop Plants with Emphasis on Rice: International Workshop on Field Screening for Drought Tolerance in Rice, ICRISAT* (eds N.P. Saxena and J.C. O'Toole), ICRISAT, Patancheru, India, pp. 128–137.

73 Crouch, J.H. and Serraj, R. (2002) DNA marker technology as a tool for genetic enhancement of drought tolerance at ICRISAT, in *Field screening for drought tolerance in crop plants with emphasis on rice: International Workshop on Field Screening for Drought Tolerance in Rice, ICRISAT,* ICRISAT, Patancheru, India.

74 Varshney, R.K., Bertioli, D.J., Moretzsohn, M.C., Vadez, V., Krishnamurthy, L., Aruna, R., Nigam, S.N., Moss, B.J., Seetha, K., Ravi, K., He, G., Knapp, S.J., and Hoisington, D.A. (2009) The first SSR-based genetic linkage map for cultivated groundnut (*Arachis hypogaea* L). *Theor. Appl. Genet.*, **118**, 729–739.

75 McCallum, C.M., Comai, L., Greene, E.A., and Henikoff, S. (2000) Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiol.*, **123**, 439–442.

76 Alonso, J.M. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 1849–11849

77 Rispail, N. (2005) Molecular and metabolic characterisation of symbiotic interactions in *Lotus japonicus*. PhD Thesis Institute of Grassland and Environmental Research (IGER), University of Wales, Aberystwyth.

78 Webb, K.J., Skot, L., Nicholson, M.N., Jorgensen, B., and Mizen, S. (2000) *Mesorhizobium loti* increases root-specific expression of a calcium-binding protein homologue identified by promoter tagging in *Lotus japonicus*. *Mol. Plant–Microbe. Interact.*, **13**, 606–616.

79 Tadege, M., Ratet, P., and Mysore, K.S. (2005) Insertional mutagenesis: a Swiss army knife for functional genomics of *Medicago truncatula*. *Trend Plant Sci.*, **10**, 229–235.

80 Baulcombe, D. (2004) RNA silencing in plants. *Nature*, **431**, 356–363.

81 Britt, A.B. and May, G.D. (2003) Re-engineering plant gene targeting. *Trend Plant Sci.*, **8**, 90–95.

82 Dalmay, T., Hamilton, A., Mueller, E., and Baulcombe, D.C. (2000) Potato virus X amplicons in *Arabidopsis* mediate genetic and epigenetic gene silencing. *Plant Cell*, **12**, 369–379.

83 Liu, Y.L., Schiff, M., and Dinesh-Kumar, S.P. (2002) Virus-induced gene silencing in tomato. *Plant J.*, **31**, 777–786.

84 Senthil-Kumar, M., Govind, G., Kang, L., Kiran-kumar, S.M., and Udayakumar, M. (2007) Functional characterization of *Nicotiana benthamiana* homologs of peanut water deficit-induced genes by virus-induced gene silencing. *Planta*, **225**, 523–539.

85 Gilchrist, E.J. and Haughn, G.W. (2005) TILLING without a plough: a new method with applications for reverse genetics. *Curr. Opin. Plant Biol.*, **8**, 211–215.

86 Henikoff, S., Till, B.J., and Comai, L. (2004) TILLING traditional mutagenesis meets functional genomics. *Plant Physiol.*, **135**, 630–636.

87 Perry, J.A., Wang, T.L., Welham, T.J., Gardner, S., Pike, J.M., Yoshida, S., and Parniske, M. (2003) A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. *Plant Physiol.*, **131**, 866–871.

88 Sharma, K.K. and Ortiz, R. (2000) Program for the application of the genetic engineering for crop improvement in the semi-arid tropics. *In Vitro Cell Dev. Biol. Plant*, **36**, 83–92.

89 Anand, R.P., Ganapathi, A., Vengadesan, G., Selvaraj, N., Anbazhagan, V.R., and Kulothungan, S. (2001) Plant regeneration from immature cotyledon-derived callus of *Vigna unguiculata* (L) Walp (cowpea). *Curr. Sci.*, **80**, 671–674.

90 Chandra, A. and Pental, D. (2003) Regeneration and genetic transformation of grain legumes: an overview. *Curr. Sci.*, **84**, 381–387.

91 Somers, D.A., Samac, D.A., and Olhoft, P.M. (2003) Recent advances in legume transformation. *Plant Physiol.*, **131**, 892–899.

92 Eapen, S. (2008) Advances in development of transgenic pulse crops. *Biotechnol. Advan.*, **26**, 162–168.

93 Tesfaye, M., Temple, S.J., Allan, D.L., Vance, C.P., and Samac, D.A. (2001) Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic

acid synthesis and confers tolerance to aluminum. *Plant Physiol.*, **127**, 1836–1844.

94 Zhang, X.H., Zhong, W.Q., and Widholm, J.M. (2005) Expression of a fungal cyanamide hydratase in transgenic soybean detoxifies cyanamide in tissue culture and in planta to provide cyanamide resistance. *J. Plant Physiol.*, **162**, 1064–1073.

95 Tarczynski, M.C., Jensen, R.G., and Bohnert, H.J. (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science*, **259**, 508–510.

96 Kavi kishore, P.B., Hong, Z., Miao, G.H., Hu, C.A.A., and Verma, D.P.S. (1995) Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.*, **108**, 1387–1394.

97 Hayashi, H., Mustardy, L., Deshnium, P., Ida, M., and Murata, N. (1997) Transformation of *Arabidopsis thaliana* with the codA gene for choline oxidase: accumulation of glycine betaine and enhanced tolerance to salt and cold stress. *Plant J.*, **12**, 133–142.

98 Kodama, H., Hamada, T., Horiguchi, G., Nishimura, M., and Iba, K. (1994) Genetic enhancement of cold tolerance by expression of a gene for chloroplast ω-3 fatty acid desaturase in transgenic tobacco. *Plant Physiol.*, **105**, 601–605.

99 Ishizaki-Nishizawa, O., Fujii, T., Azuma, M., Sekiguchi, K., Murata, N., Ohtani, T., and Toguri, T. (1996) Low-temperature resistance of higher plants is significantly enhanced by a nonspecific cyanobacterial desaturase. *Nat. Biotechnol.*, **14**, 1003–1006.

100 Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D., and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.*, **110**, 249–257.

101 McKersie, B.D., Bowley, S.R., Harjanto, E., and Leprince, O. (1996) Water-deficit tolerance and field performance of transgenic alfalfa over-expressing superoxide dismutase. *Plant Physiol.*, **111**, 1177–1181.

102 Holmstrom, K.O., Somersalo, S., Mandal, A., Palva, E.T., and Welin, B. (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. *J. Exp. Bot.*, **51**, 177–185.

103 Delauney, A.J. and Verma, D.P.S. (1993) Proline biosynthesis and osmoregulation in plants. *Plant J.*, **4**, 215–223.

104 Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Lett.*, **461**, 205–210.

105 Zhu, B., Su, J., Chang, M., Verma, D.P.S., Fan, Y.L., and Wu, R. (1998) Overexpression of delta1-pyrroline-5-carboxylate synthase gene and analysis of tolerance to water and salt stress in transgenic rice. *Plant Sci.*, **199**, 41–48.

106 Yamada, M., Morishita, H., Urano, K., Shiozaki, N., Yamaguchi-Shinozaki, K., Shinozaki, K., and Yoshiba, Y. (2005) Effects of free proline accumulation in petunias under drought stress. *J. Exp. Bot.*, **56**, 1975–1981.

107 Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J.-K. (1997) Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell*, **9**, 1935–1949.

108 Bhatnagar-Mathur, P., Vadez, V., and Sharma, K.K. (2008) Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. *Plant Cell Rep.*, **27**, 411–424.

109 Bhatnagar-Mathur, P., Jyostna Devi, M., Vadez, V., and Sharma, K.K. (2009) Differential antioxidative responses in transgenic peanut bear no relationship to their superior transpiration efficiency under drought stress. *J. Plant Physiol.*, **166**, 1207–1217.

110 Bohnert, H.J., Nelson, D.E., and Jensen, R.G. (1995) Adaptations to environmental stresses. *Plant Cell*, **7**, 1099–1111.

111 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress inducible transcription factor. *Nat. Biotechnol.*, **17**, 287–291.

112 Bhatnagar-Mathur, P., Devi, M.J., Srinivas Reddy, D., Lavanya, M., Vadez, V., Serraj, R., Yamaguchi-Shinozaki, K., and Sharma, K.K. (2007) Stress-inducible expression of AtDREB1A in transgenic peanut (*Arachis hypogaea* L) increases transpiration efficiency under water-limiting conditions. *Plant Cell Rep.*, **26**, 2071–2082.

113 Hinchee, M.A.W., Connor-Ward, D.V., Newell, C.A., McDonnell, R.E., Sato, S.J., Gasser, C.S., Fischhoff, D.A., Re, D.B., Fraley, R.T., and Horsch, R.B. (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. *Nat. Biotechnol.*, **6**, 915–922.

114 McCabe, D.E., Swain, W.F., Martinell, B.J., and Christou, P. (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. *Nat. Biotechnol.*, **6**, 923–926.

115 James, C. (2003) Global review of commercialized transgenic crops. *Curr. Sci.*, **84**, 303–309.

116 Christou, P. (1997) Biotechnology applied to grain legumes. *Field Crops Res.*, **53**, 83–97.

117 Trick, H.N., Dinkins, R.D., Santarem, E.R., Samaloyov, R.D.V., Meurer, C., Walker, D., Parrott, W.A., Finer, J.J., and Collins, G.B. (1997) Recent advances in soybean transformation, *Plant Tissue Cult. Biotechnol*, **3**, 9–26.

118 Chee, P.P. and Hu, C.-Y. (2000) Transgenic soybean (*Glycine max*), in *Biotechnology in Agriculture and Forestry*, vol. 46 (ed. Y.P.S. Bajaj), Transgenic Crops I, Springer Verlag, pp. 268–282.

119 Xue, R. and Zhang, B. (2007) Increased endogenous methyl jasmonate altered leaf and root development in transgenic soybean plants. *J. Genet. Genomics*, **34**, 339–346.

120 Xue, R.-G., Zhang, B., and Xie, H.-F. (2007) Overexpression of a NTR1 in transgenic soybean confers tolerance to water stress. *Plant Cell Tissue Organ Cult.*, **89**, 177–183.

121 Sharmila, P., Phanindra, M.L.V., Anwar, F., Singh, K., Gupta, S., and Pardha Saradhi, P. (2009) Targeting prokaryotic choline oxidase into chloroplasts enhance the potential of photosynthetic machinery of plants to withstand oxidative damage. *Plant Physiol. Biochem.*, **47**, 391–396.

122 Bhatnagar-Mathur, P., Vadez, V., Jyostna Devi, M., Lavanya, M., Vani, G., and Sharma, K.K. (2009) Genetic engineering of chickpea (*Cicer arietinum* L.) with the *P5CSF129A* gene for osmoregulation with implications on drought tolerance. *Mol. Breed.*, **23**, 591–606.

123 Turner, N.C. and Jones, M.M. (1980) Turgor maintenance by osmotic adjustment: a review and evaluation, in *Adaptation of Plants to Water and High Temperature Stress* (eds N.C. Turner and P.J. Kramer), Wiley Interscience, New York, pp. 38–42.

124 Morgan, J.M. (1984) Osmoregulation and water stress in higher plants. *Annu. Rev. Plant Physiol.*, **35**, 299–348.

125 Serraj, R. and Sinclair, T.R. (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? *Plant Cell Environ.*, **25**, 333–341.

126 Behnam, B., Kikuchi, A., Celebi-Toprak, F., Yamanaka, S., Kasuga, M., Yamaguchi-Shinozaki, K., and Watanabe, K.N. (2006) The *Arabidopsis DREB1A* gene driven by the stress-inducible *rd29A* promoter increases salt-tolerance in proportion to its copy number in tetrasomic tetraploid potato (*Solanum tuberosum*). *Plant Biotechnol.*, **23**, 169–177.

127 Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998) *Arabidopsis* CBF1 over-expression induces COR genes and enhances freezing tolerance. *Science*, **280**, 104–106.

128 Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) Two transcription

factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*, **10**, 1391–1406.

129 Sanan-Mishra, N., Pham, X.H., Sopory, S.K., and Tuteja, N. (2005) Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. *Proc. Natl. Acad. Sci. USA*, **102**, 509–514.

130 Singla-Pareek, S.L., Reddy, M.K., and Sopory, S.K. (2003) Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. *Proc. Natl. Acad. Sci. USA*, **100**, 14672–14677.

131 Wan, X.R. and Li, L. (2006) Regulation of ABA level and water-stress tolerance of *Arabidopsis* by ectopic expression of a peanut 9-*cis*-epoxycarotenoid dioxygenase gene. *Biochem. Biophys. Res. Commun.*, **347** (4), 1030–1038.

132 Peng, H., Yu, X.W., Cheng, H., Shi, Q., Zhang, H., Li, J., and Ma, H. (2010) Cloning and characterization of a novel NAC family gene CarNAC1 from chickpea (*Cicer arietinum* L). *Mol. Biotechnol.*, **44**, 30–40.

133 Bhattarai, T. and Fettig, S. (2005) Isolation and characterization of a dehydrin gene from *Cicer pinnatifidum*: a drought-resistant wild relative of chickpea. *Physiol. Plant.*, **123**, 452–458.

134 Shukla, R.K., Raha, S., Tripathi, V., and Chattopadhyay, D. (2006) Expression of CAP2, an APETALA2-family transcription factor from chickpea, enhances growth and tolerance to dehydration and salt stress in transgenic tobacco. *Plant Physiol.*, **142**, 113–123.

135 Chen, M., Wang, Q.Y., Cheng, X.G., Xu, Z.S., Li, L.C., Ye, X.G., Xia, L.Q., and Ma, Y.Z. (2007) *GmDREB2*, a soybean DRE-binding transcription factor, conferred drought and high-salt tolerance in transgenic plants. *Biochem. Biophys. Res. Commun.*, **353**, 299–305.

136 Chen, M., Xu, Z., Xia, L., Li, L., Cheng, X., Dong, J., Wang, Q., and Ma, Y. (2009) Cold-induced modulation and functional analyses of the DRE-binding transcription factor gene, GmDREB3, in soybean (*Glycine max* L.). *J. Exp. Bot.*, **60**, 121–135.

137 Jin, T., Chang, Q., Li, W., Yin, D., Li, Z., Wang, D., Liu, B., and Liu, L. (2010) Stress-inducible expression of GmDREB1 conferred salt tolerance in transgenic alfalfa. *Plant Cell Tissue Organ Cult.*, **100**, 219–227.

138 Li, X.P., Tian, A.G., Luo, G.Z., Gong, Z.Z., Zhang, J.S., and Chen, S.Y. (2005) Soybean DRE-binding transcription factors that are responsive to abiotic stresses. *Theor. Appl. Genet.*, **110**, 1355–1362.

139 Zhang, G., Chen, M., Chen, X., Xu, Z., Guan, S., Li, L.-C., Li, A., Guo, J., Mao, L., and Ma, Y. (2008) Phylogeny, gene structures, and expression patterns of the ERF gene family in soybean (*Glycine max* L.). *J. Exp. Bot.*, **59**, 4095–4107.

140 Liao, Y., Zou, H.F., Wang, H.W., Zhang, W.K., Ma, B., Zhang, J.S., and Chen, S.Y. (2008) Soybean GmMYB76, GmMYB92, and GmMYB177 genes confer stress tolerance in transgenic *Arabidopsis* plants. *Cell Res.*, **18**, 1047–1060

141 Liao, Y., Zou, H.F., Wei, W., Hao, Y.J., Tian, A.G., Huang, J., Liu, Y.F., Zhang, J.S., and Chen, S.Y. (2008) Soybean GmbZIP44, GmbZIP62 and GmbZIP78 genes function as negative regulator of ABA signaling and confer salt and freezing tolerance in transgenic *Arabidopsis*. *Planta*, **228**, 225–240

142 Zhou, Q.Y., Tian, A.G., Zou, H.F., Xie, Z.M., Lei, G., Huang, J., Wang, C.M., Wang, H.W., Zhang, J.S., and Chen, S.Y. (2008) Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic *Arabidopsis* plants. *Plant Biotechnol. J.*, **6**, 486–503.

143 Cheng, L., Huan, S., Sheng, Y., Hua, X., Shu, Q., Song, S., and Jing, X. (2009) GMCHI, cloned from soybean [*Glycine max* (L.) Meer.], enhances survival in transgenic *Arabidopsis* under abiotic stress. *Plant Cell Rep.*, **28**, 145–153.

144 Xie, Z.M., Zou, H.-F., Lei, G., Wei, W., Zhou, Q.-Y., Niu, C.-F., Liao, Y., Tian, A.-G., Ma, B., Zhang, W.-K.,

Zhang, J.-S., and Chen, S-Y. (2009) Soybean trihelix transcription factors GmGT-2A and GmGT-2B improve plant tolerance to abiotic stresses in transgenic *Arabidopsis*. *PLoS ONE* **4**, e6898.

145 Wei, W., Huang, J., Hao, Y.J., Zou, H.F., Wang, H.W., Zhao, J.Y., Liu, X.Y., Zhang, W.K., Ma, B., Zhang, J.S., and Chen, S.Y. (2009) Soybean GmPHD-type transcription regulators improve stress tolerance in transgenic *Arabidopsis* plants. *PLoS One*, **4**, e7209.

146 Rodrigues, S.M., Andrade, M.O., Gomes, A.P.S., DaMatta, F.M., Baracat-Pereira, M.C., and Fontes, E.P.B. (2006) *Arabidopsis* and tobacco plants ectopically expressing the soybean antiquitin-like ALDH7 gene display enhanced tolerance to drought, salinity, and oxidative stress. *J. Exp. Bot.*, **57**, 1909–1918.

147 Miflin, B. (2000) Crop improvement in the 21st century. *J. Exp. Bot.*, **51**, 1–8.

148 Vadez, V., Krishnamurthy, L., Kashiwagi, J.W., Kholova, J., Devi, J.M., Sharma, K.K., Bhatnagar-Mathur, P., Hoisington, D.A., Hash, C.T., Bidinger, F.R., and Keatinge, J.D.H. (2007) Exploiting the functionality of root systems for dry, saline, and nutrient deficient environments in a changing climate. *J. SAT Agric. Res.*, **4** (Special Symposium edition). DOI: 10.3914/ICRISAT.0099.

149 Ritchie, J.T. (1981) Water dynamics in the soil–plant–atmosphere system. *Plant Soil*, **58**, 81–96.

150 Sinclair, T.R. and Ludlow, M.M. (1985) Who taught plants thermo-dynamics? The unfilled potential of plant water potential. *Aust. J. Plant Physiol.*, **12**, 213–217.

151 Sinclair, T.R. and Ludlow, M.M. (1986) Influence of soil water supply on the plant water balance of four tropical grain legumes. *Aust. J. Plant Physiol.*, **13**, 329–341.

152 Sadras, V.O. and Milroy, S.P. (1996) Soil–water thresholds for the responses of leaf expansion and gas exchange. *Field Crops Res.*, **47**, 253–266.

153 Kholova, J., Hash, C.T., Kakkera, A., Kocova, M., and Vadez, V. (2010) Constitutive water conserving mechanisms are correlated with the terminal drought tolerance of pearl millet (*Pennisetum americanum* L.). *J. Exp. Bot.*, **61**, 369–377.

154 Kholova, J., Hash, C.T., Lava Kumar, P., Yadav, R.S., Kakkera, A., Kocova, M., and Vadez, V. (2010) Terminal drought tolerant pearl millet [*Pennisetum glaucum* (L.) R. Br.] have high leaf ABA and limit transpiration at high vapor pressure deficit. *J. Exp. Bot.*, **61**. doi: 10.1093/jxb/erq013

155 Vadez, V., Rao, S., Kholova, J., Krishnamurthy, L., Kashiwagi, J., Ratnakumar, P., Sharma, K.K., Bhatnagar-Mathur, P., and Basu, P.S. (2008) Roots research for legume tolerance to drought: *Quo vadis?* *J. Food Legum.*, **21**, 77–85.

156 Eujayl, I., Erskine, W., Baum, M., and Pehu, E. (1999) Inheritance and linkage analysis of frost injury in lentil. *Crop Sci.*, **39**, 639–642.

157 Kahraman, A., Kusmenoglu, I., Aydin, N., Aydogan, A., Erskine, W., and Muehlbauer, F.J. (2004) QTL mapping of winter hardiness genes in lentil. *Crop Sci.*, **44**, 13–22.

158 Kassem, M.A., Meksem, K., Kang, C.H., Njiti, V.N., Kilo, V., Wood, A.J., and Lightfoot, D.A. (2004) Loci underlying resistance to manganese toxicity mapped in a soybean recombinant inbred line population of "Essex" × "Forrest". *Plant Soil*, **260**, 197–204.

159 VanToai, T.T., St Martin, S.K., Chase, K., Boru, G., Schnipke, V., Schmitthenner, A.F., and Lark, K.G. (2001) Identification of a QTL associated with tolerance of soybean to soil waterlogging. *Crop Sci.*, **41**, 1247–1252.

160 Li, Y.D., Wang, Y.J., Tong, Y.P., Gao, J.G., Zhang, J.S., and Chen, S.Y. (2005) QTL mapping of phosphorus deficiency tolerance in soybean (*Glycine max* L Merr). *Euphytica*, **142**, 137–142.

161 Sledge, M.K., Bouton, J.H., Dall'Agnoll, M., Parrott, W.A., and Kochert, G. (2002) Identification and confirmation of aluminum tolerance QTL in diploid *Medicago sativa* subsp *coerulea*. *Crop Sci.*, **42**, 1121–1128.

162 Zhang, J.-Y., Broeckling, C.D., Blancaflor, E.B., Sledge, M.K., Sumner, L.W., and Wang, Z.-Y. (2005) Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *Plant J.*, **42**, 689–707.

163 McKersie, B.D., Murnaghan, J., Jones, K.S., and Bowley, S.R. (2000) Iron-superoxide dismutase expression in transgenic alfalfa increases winter survival without a detectable increase in photosynthetic oxidative stress tolerance. *Plant Physiol*, **122**, 1427–1437.

164 McKersie, B.D., Bowley, S.R., and Jones, K.S. (1999) Winter survival of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.*, **119**, 839–847.

165 McKersie, B.D., Chen, Y., de Beus, M., Bowley, S.R., Bowler, C., Inze, D., Halluin, K.D., and Botterman, J. (1993) Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medicago sativa* L.). *Plant Physiol.*, **103**, 1155–1163.

166 Alvim, F.C., Carolino, S.M.B., Cascardo, J.C.M., Nunes, C.C., Martinez, C.A., Otoni, W.C., and Fontes, E.P.B. (2001) Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. *Plant Physiol.*, **126**, 1042–1054.

167 Kim, J.C., Lee, S.H., Cheong, Y.H., Yoo, C.-M., Lee, S.I., Chun, H.J., Yun, D.-J., Hong, J.C., Lee, S.Y., Lim, C.O., and Cho, M.J. (2001) A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. *Plant J.*, **25**, 247–259.

168 Winicov, I. and Bastola, D.R. (1999) Transgenic overexpression of the transcription factor *Alfin1* enhances expression of the endogenous *MsPRP2* gene in alfalfa and improves salinity tolerance of the plant. *Plant Physiol.*, **120**, 473–480.

169 Oberschall, A., Deak, M., Torok, K., Sass, L., Vass, I., Kovacs, I., Feher, A., Dudits, D., and Horvath, G.V. (2000) A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stress. *Plant J.*, **24**, 437–446.

170 Merchan, F., Breda, C., Hormaeche, J.P., Sousa, C., Kondorosi, A., Aguilar, O.M., Megias, M., and Crespi, M. (2003) A Krüppel-like transcription factor gene is involved in salt stress responses in *Medicago* spp. *Plant Soil*, **257**, 1–9.

171 Zhang, J.-Y., Broeckling, C.D., Sumner, L.W., and Wang, Z.-Y. (2007) Heterologous expression of two *Medicago truncatula* AP2 domain transcription factor genes, WXP1 and WXP2, in *Arabidopsis* led to increased leaf wax accumulation and improved drought tolerance, but differential response in freezing tolerance. *Plant Mol. Biol.*, **64**, 265–278.

172 Rodriguez-Uribe, L. and O'Connell, M.A. (2006) A root-specific bZIP transcription factor is responsive to water deficit stress in tepary bean (*Phaseolus acutifolius*) and common bean (*P. vulgaris*). *J. Exp. Bot.*, **57**, 1391–1398.

173 Guo, Y. and Gan, S. (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. *Plant J.*, **46**, 60–612.

40
Fruit Crops: Omic Approaches toward Elucidation of Abiotic Stress Tolerance

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Abiotic stresses are likely to affect yield potential of crop plants greatly and globally in next 50–100 years by way of inevitable increment in atmospheric CO_2 concentration, salinity, drought, heat, flooding, chemicals in soil, and so on. These stresses affect the whole plant resulting in a stress response wherein plants divert their resources toward adapting to changed environment. This entails a major change in their gene expression, proteins, metabolites, and so on. There is an urgent need to understand how plants respond to these stresses and adapt for their survival. In order to address this problem, it is imperative to understand the global changes occurring in plants at all levels during stress condition. Omics approaches offer such tools that can analyze changes at genomic, transcriptomic, proteomic, and metabolomic levels. Such studies have primarily been carried out in model plants such as *Arabidopsis*, rice, *Populus*, and so on whereas information on fruit crop is lacking. We review here the progress made in understanding various stress responses in fruit crops using one or the other omics approaches. With the availability of whole-genome sequences of tomato, papaya, and grape, and whole transcriptome of tomato, grape berries, grapefruit, apple, and peach initiated, it should be possible to decipher gene regulation during stress response in fruit crops. Proteome and metabolome analyses of few fruits are also in progress, which should help answer several important questions regarding stress response in fruit plants. With the availability of these data, efforts are being made to identify genes and pathways that can be engineered to produce transgenic fruit crops resistant to abiotic stress.

40.1
Introduction

Plants have much higher genetic potential for yield than we realize. This unrealized potential could be attributed mainly due to environmental conditions that do not

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support their full yield potential. This has been substantiated time and again by examining native or wild cultivars against domesticated ones [1]. Abiotic stresses are one of the major causative factors that affect plant yield. Drought, salinity, cold, heat, flooding, and chemicals are the most common abiotic stresses that affect plant growth, development, and its yield potential. These stresses have been shown to either bring about osmotic stress or oxidative stress or both that disrupts functional and/or structural proteins and membranes [2]. Salinization of arable land is increasing and expected to result in 50% land loss by 2050; greenhouse effect that is contributing to global warming is expected to increase temperature by 3–5 °C in the next 50–100 years resulting in higher frequencies of heat waves, tropical cyclones, floods, and prolonged drought conditions [3]. These climate changes are surely to affect crop nutrition, efficient water uptake, and finally the yield [4]. In order to address these issues vis-à-vis yield potential, a careful combination of various approaches need to be applied to improve significantly the abiotic stress tolerance in crop plants. Molecular biology has deciphered several fundamental questions and we are now beginning to understand that acclimatization of plants to abiotic stress conditions is a highly complex and coordinated process involving recruitment of several hundred genes and a strong signaling system. Strategic use of novel genes for transfer could be one of the key factors in developing crop plants resistant to abiotic stress. Recently, many plant genomes have been sequenced and others are in progress. Interestingly, 20–40% genes have been accounted for no known functions [5]. Many of these are species specific making them potential candidates for stress adaptability [6]. Omic approaches have now become inevitable not only in elucidating the regulation of various pathways and gene functions but also in assigning a specific role to a gene, its possible usage, and its biotechnological feasibility. Genomics, transcriptomics, proteomics, and metabolomics are some of the modern tools in biology that have been introduced recently and utilize high-end equipment to carry out these analyses in a short period of time with high accuracy. How plants respond to various abiotic stresses by way of their expression at genome, transcriptome, proteome, and metabolome levels has been depicted in Figure 40.1. The application of these omic approaches either singly or in combination has answered several questions of plant biology paving the way for future research.

Most omic approaches have so far been applied to major crop plants both cereals and legumes or model plants like *Arabidopsis*. In this chapter, we have tried to review the recent progress in omic approaches that have been applied to understand the mechanism of stress tolerance in fruit crops including tomato. Though exact data are not available, substantial losses in fruit crops occur due to abiotic stress conditions the world over. For example, deficit irrigation (DI) to mandarin plants could reduce fruit yield up to 40% in some cultivars, whereas only 27% reduction was observed in some other cultivars depending upon their rootstock [7]. Not only yield but also the quality of fruit may be affected due to abiotic stress condition. When strawberry plants were irrigated differently with various concentrations of saline, they differed in their antioxidant capacity and other biochemical contents such as glutathione, phenol, anthocyanin, and so on [8]. There are many other studies as well indicating

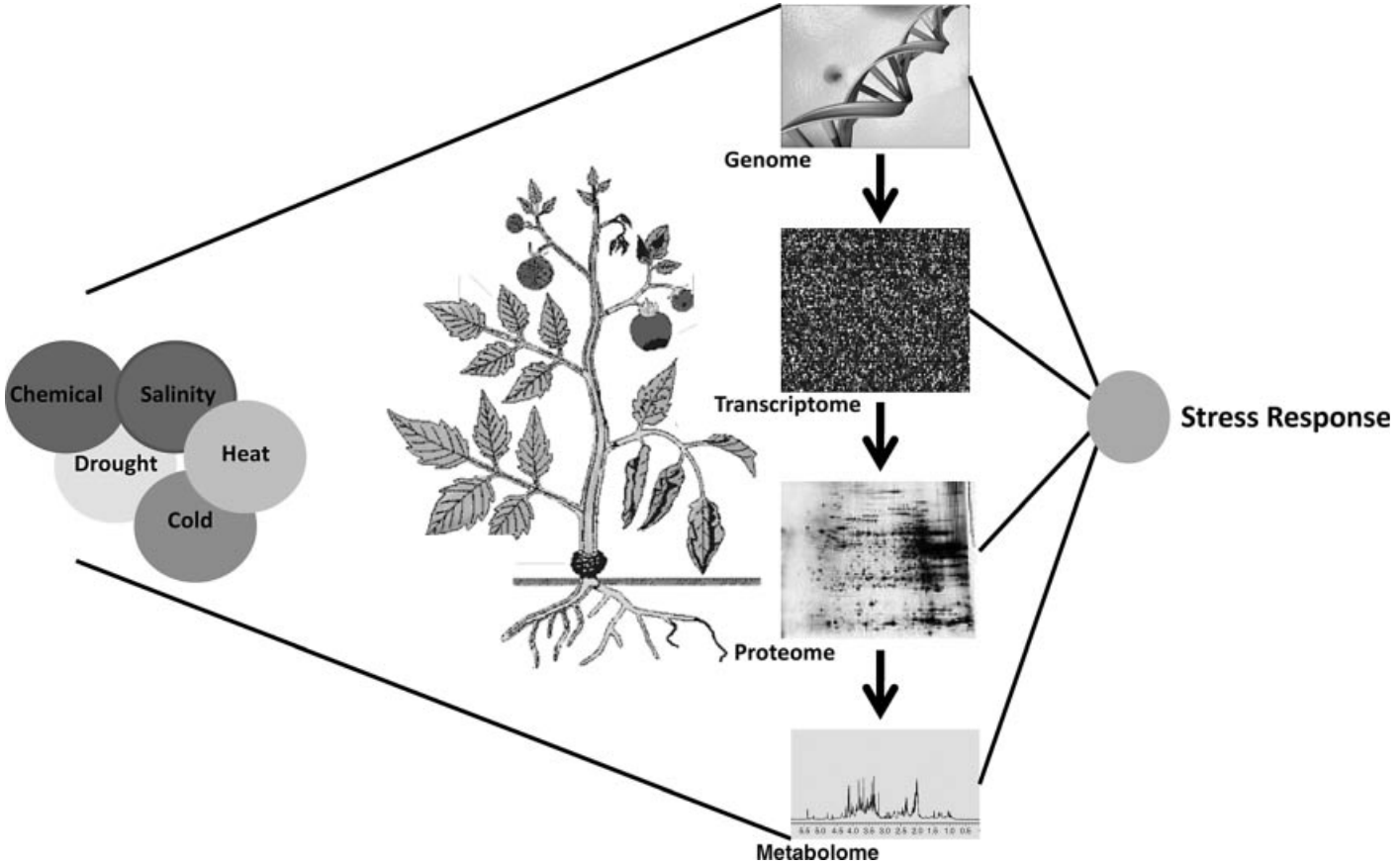

Figure 40.1 Schematic representation of various abiotic stresses affecting whole plant, which may alter homeostatic balance at various levels such as genome, transcriptome, proteome, and metabolome. These alterations together constitute stress response.

that yield loss in fruit crop due to abiotic stress conditions could be very high. In the following sections, we have reviewed the recent status of omic approaches in elucidating stress-related response of fruit plants.

40.2 Genomics and Transcriptomics of Fruit Crops

The depiction of entire genome of at least papaya, grape, and tomato has been completed and it is likely that the genomes of banana and apple will be available soon. The project on citrus fruit genome has already been initiated by the USDA and will be a part of Tree Fruit Genome Database. Work on identifying various QTL including those related to stress is in progress. Genes that are involved in chromatin modeling and epigenetic control are also being identified [9]. However, except in the case of tomato [10], not much progress has been made in other fruits with respect to identification of various QTL related to stress. This could be due to the fact that the sequences became available only recently and mostly as draft sequences. The annotations are still being carried out and we hope to see some good progress in coming years. However, with the advent of high-throughput technologies, the global analysis of the transcriptome is possible and being carried out in some cases. Out of that, the microarray analysis is one such technique that gives us an idea of the global transcriptome profile during any event/treatment. At the moment, the microarray chips for fruit species are available only for tomato, *Vitis vinifera*, and citrus fruits. Other techniques such as differential display, subtractive hybridization, and now transcriptome sequencing are being employed to study whole transcriptome profiles of species for which the genome sequence is not known. In this chapter, we have reviewed how certain genes that are related to abiotic stress have been identified by various researchers utilizing one or the other available technique.

The response to abiotic stresses such as heat, cold, drought, salinity, and so on in fruit crops is mediated by several genes and proteins. Prominent among these are transcription factors of the AP2 domain/ERF family that are activated in these stresses. Studies in recent years have led to identification of ERFs in tomato such as DREB3 that was shown to be activated by salinity, heat, and cold [11]. Another ERF, the JERF3, showed early response to both cold and salt within 10 min of respective treatments [12]. The tomato TERF1 gene was also identified as a salt-responsive gene [13]. The pepper ERF gene CaERFLP1 is induced by salt and in tomato the LeCBF1 gene, another AP2/DREB-type gene and a homologue of *Arabidopsis* CBF1 gene, was induced within 30 min of cold treatment although overexpression of the gene in tomato did not impart cold tolerance to plants [14]. Another transcription factor, SlAREB1 encoding a bZIP transcription factor, was induced in response to salt, cold, drought, and ABA [15]. The phenylalanine ammonia lyase gene (SlPAL5) was also shown to be expressed in response to salinity, cold, and osmotic stress. Besides transcription factors, several other genes have been identified that respond to one or the other stresses.

Cold stress is one of the commonly endured stresses by fruit plants, either during development or during storage. Many of the commercially available fruits are susceptible to cold stress resulting in major economic losses. Whole-transcriptome analysis in tomato, grape berries, grapefruit, apple, and peach has shown the involvement of genes related to various metabolic pathways to be regulating cold stress. Weiss and Egea-Cortines [16] characterized the effect of cold stress (6 °C for 48 h) in tomato fruits (MicroTom) during ripening using subtractive PCR. In this study, they identified many housekeeping genes that were upregulated during cold stress, such as genes involved in protein synthesis (25S ribosomal RNA, ribosomal proteins L10, L27, S11, S13, and an rRNA-encoded homing endonuclease), actins, senescence-associated protein (SGN-U43780), and genes related to carbohydrate metabolism (starch phosphorylase (SGN-U148218), triosephosphate isomerase (SGN-U145276), oraldo/keto reductase (SGN-U179653)). Several chloroplast (chlorophyll A–B binding protein SGN-U69362) and mitochondrial encoded genes (ATPsynthase beta chain SGN-U106411) were also upregulated by the cold stress. Among the known genes, dehydrin was found to be upregulated in cold stress; this dehydrin clustered with cold-induced dehydrin genes from other species. A study on the chilling effect on grapefruit during storage [17] showed a large number of genes and many pathways to be affected. Chilling stress (5 °C) for 2 weeks resulted in differential expression of 7500 genes with significant p values (≤ 0.005), and induction or repression of transcript levels to at least fourfold. They also studied the effect of preconditioning of chilling at 16 °C before transferring to 5 °C. This led to the identification of a chilling regulon. This group of transcripts defined the basic transcripts involved in natural responses of citrus to low temperature. In the chilling regulon, a massive downregulation of transcripts related to cell wall and defense against pathogens, photosynthesis, respiration, protein metabolism, DNA and RNA metabolism, secondary metabolism, water channels, and senescence was identified. They also found that chilling activated various adaptation processes resulting in significant changes in transcripts encoding membrane proteins, lipid, and sterol metabolism, carbohydrate metabolism, stress stimulus, hormone biosynthesis, and DNA binding and transcription factors. Study of leaf, bark, xylem, and root tissue cDNA libraries from Royal Gala apples in control, drought, and cold stress showed significant changes in gene expression [18]. In the case of cold stress libraries, changes in gene expression were observed in dehydrin and metallothionein-like proteins, ubiquitin proteins, a dormancy-associated protein, a plasma membrane intrinsic protein, and an RNA binding protein. A large number of unique genes expressed in the leaf in response to cold were also observed in this study.

A recent study on transcriptome profiling of banana during drought stress was done by cross hybridization using rice Affymetrix gene chip [19]. A total of 2910 transcripts, with a more than twofold differential expression, were observed. Many of the differentially regulated genes identified in this study have been known to be involved in biotic and abiotic stresses in other plant species as well. The differentially regulated transcription factors were from DREB, ERF, MYB, BZIP, and bHLH families. Fifty-two drought-sensitive transcripts were homologous for QTL associated with drought tolerance in rice too. The categories of differentially regulated

genes belong to cellular metabolism, response to biotic and abiotic stresses, protein metabolism, transcription, transport signal transduction, and so on. In a study on anthocyanin biosynthesis during drought stress in grapevines (*V. vinifera* L.), it was found that there was a strong correlation between the gene expression of anthocyanin pathway and the anthocyanin content [20]. The expression of the genes encoding enzymes of the flavonoid pathway and related transcription factors were studied. The authors noted that at least 84% of the total variation in anthocyanin content was explained by the linear relationship between the integral of mRNA accumulation of the specific anthocyanin biosynthetic gene UDP-glucose:flavonoid 3-*O*-glucosyl-transferase (UFGT) and the metabolite content during time series from véraison through ripening. The genes of the flavonoid pathway chalcone synthase (CHS2, CHS3) and flavanone 3-hydroxylase (F3H) also showed high correlation. The genes coding for flavonoid 3′,5′-hydroxylase (F3′5′H) and *O*-methyltransferase (OMT) were also upregulated in berries from dehydrated plants. The authors suggested that the interrelationship between developmental and environmental signaling pathways was magnified during water deficit. In this case, water stress induced genes of flavonoid and brassinosteroid pathway that actively promotes fruit maturation and anthocyanin biosynthesis. The whole-transcriptome analysis of Royal Gala apple fruit tree also suggests the upregulation of genes related to stress categories such as heat shock, dehydrins, peroxidases, and lipid transfer protein photosynthesis [18].

40.3
Proteomics of Fruit Crops

Proteomic studies of plant response to abiotic stress include analyses of the effects of high light, extreme temperatures, water deficit, salinity, and the presence of heavy metals or toxic chemicals in the environment. Study of proteomics has allowed the identification of novel genes and the characterization of their regulation and function. Some of the high-end techniques have made proteomic studies possible with a high degree of accuracy. Mass spectrometry (MS) is one powerful method for the characterization of proteins. Intact proteins are ionized by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) and then introduced to a mass analyzer using either time-of-flight (TOF) MS or Fourier transform ion cyclotron resonance (FT-ICR). These two types of instruments are preferable because of their wide mass range and mass accuracy. Shotgun proteomics is another method of identifying proteins in complex mixtures using a combination of HPLC with mass spectrometry. In shotgun proteomics, the proteins in the mixture are digested and the resulting peptides are separated by liquid chromatography (LC). Tandem mass spectrometry is then used to identify the peptides. Prior to mass spectrometry, proteins are fractionated by 2D electrophoresis.

In recent years, proteomics has been proven to be a powerful method to explore the protein changes in fruit during development and ripening [21–23]. On the other hand, there is still a lack of information about harvested fruit senescence and fruits under abiotic stress using proteomic analysis. Proteomic analysis is difficult in fruits

where protein content is less and carbohydrates are in abundance. In addition, studies in fruits are also restricted due to annual reproductive cycle of most plants. Also, proteomics relies on availability of genomic sequence data for use in protein identification and this information is lacking for most of the fruits [24]. Despite various difficulties, good work on fruit proteomics has been published in recent years.

A comparative proteomic approach has been adopted in combination with physiological and biochemical analysis of tomato leaves responding to waterlogging stress. Waterlogging resulted in increases in relative ion leakage, lipid peroxidation and *in vivo* H_2O_2 content, whereas the chlorophyll content was decreased [25]. A total of 52 protein spots were differentially expressed, wherein 33 spots were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry or electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis. A number of novel proteins were differentially expressed or appeared only in the PEG-fractionated protein samples, indicating that PEG fractionation system can be used as a versatile protein fractionation technique in proteomic analysis to identify novel or low-abundant proteins from all kinds of plant species. The identified proteins are involved in several processes, namely, photosynthesis, disease resistance, stress and defense mechanisms, and protein biosynthesis. On the basis of their results, Ahsan *et al.* [25] suggested that transcription levels are not always concomitant to the translation level.

Growth inhibition in acid soils due to Al stress affects crop production worldwide. To understand mechanisms in sensitive crops that are affected by Al stress, a proteomic analysis of primary tomato root tissue, grown in Al-amended and non-amended liquid cultures, was performed by Zhou *et al.* [26]. DIGE-SDS-MALDI-TOF-TOF analysis of these tissues resulted in the identification of 49 proteins that were differentially accumulated. Most of these proteins were of defense and detoxification category. Proteomic analysis of shade-avoidance response in tomato leaves was carried out by Hattrup *et al.* [27]. Protein expression differences were investigated using two-dimensional differential in-gel electrophoresis and nanoflow high-performance liquid chromatography–tandem mass spectrometry. A large group of proteins related to metabolism and respiration were greatly reduced in accumulation in shade-grown plants, and there was also evidence of significant proteolysis occurring. Four stress-related proteins appeared to be constitutively expressed as a result of heat acclimatization, while three distinct stress-related proteins accumulated as part of the shade-avoidance response.

The characterization of proteins isolated from skin tissue is apparently an essential parameter for understanding grape ripening. Therefore, proteome profile of grape skin during maturation was developed by Lucker *et al.* [28]. Three different stages of ripening were taken to assess protein distribution in grape skin during ripening. Proteome analysis revealed that proteins involved in photosynthesis, carbohydrate metabolisms, and stress response were being overexpressed at the beginning of color change; however, at the end of color change, proteins involved in anthocyanin synthesis were overexpressed. Proteins involved in defense mechanisms like chitinase and b-1,3-glucanase isoforms were prevalent at the time of fruit harvest. The

differences observed in proteome maps showed that significant metabolic changes occur in grape skin during this crucial phase of ripening.

Grimplet *et al.* [29] studied the changes in protein expression in grape berry tissues during drought. The authors carried out comparative 2D-PAGE analysis on grape pulp, skin, and seed under well-watered and water deficit conditions. Water deficit condition altered the levels of approximately 7% of skin and pulp proteins, but no significant change was observed on seed protein expression. In skin, high levels of peptidases and α and β subunits of proteasome indicated that drought stress increases protein turnover in grape. Selected enzymes for flavonoid biosynthesis and reactive oxygen detoxification enzymes were also more abundant in berry skin of grapes grown under water deficient condition, whereas grape flesh showed increased levels of enzymes such as isoflavone reductase, glutamate decaroxylase, and endo-chitinase. Comparison of mRNA levels and protein expression patterns suggested that posttranscriptional processes govern protein accumulation.

Lara *et al.* [30] studied the effect of high temperature both on peach quality and on carbon metabolism. A comparative analysis of the peach mesocarp proteome variations was carried out using 2D differential gel electrophoresis (2D-DIGE) and several candidate proteins were identified that might be correlated with chilling injury symptoms. Lara *et al.* [30] detected 57 differentially expressed proteins; 67% of these were of defense and stress response categories. High temperature repressed polyphenol oxidase and ascorbate peroxidase. These enzymes are involved in browning process during chilling injury in peach, which reduces taste and nutritional value of the fruit. Qin *et al.* [31] worked on mitochondrial proteome during fruit senescence. On the basis of their studies on apple, they concluded that fruit senescence is correlated with the dynamic alterations in the mitochondrial proteome. Mitochondrial proteins involved in carbon metabolism, and stress response, tricarboxylic acid cycle, and electron transport chain were found differentially expressed during fruit senescence. The levels of many mitochondrial proteins were significantly reduced during oxidative stress. The group analyzed the mitochondrial proteome variations upon exposure to high oxygen (100%) to understand the regulatory effect of ROS in senescence. Activity of manganese superoxide dismutase was reduced after high oxygen exposure, accompanied by an increase in oxidative protein carbonylation (damaged proteins). A novel protein, mitochondrial porin, was downregulated during oxidative stress and its dysfunction was directly correlated with impairment of mitochondrial function. Proteome data obtained for apple senescence suggested that ROS might regulate fruit senescence by changing expression profiles of specific mitochondrial proteins and impairing the biological function of these proteins.

Wang *et al.* [32] worked on climacteric fruit Jujube common in China. They found that oxalic acid (OA) at the concentration of 5 mM could delay Jujube fruit senescence by reducing ethylene production, repressing fruit reddening, and reducing alcohol content. They used a proteomics approach to compare soluble proteome of Jujube fruits treated with water or 5 mM OA for 10 min. A total of 25 differentially expressed proteins were identified by using electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF-MS/MS). Analyses of differentially

expressed proteins suggested that OA treatment might affect ethanol and ethylene metabolism, resulting in delay in senescence and increase in resistance of Jujube fruits against fungal pathogen as alcohol dehydrogenase and 1-aminocyclopropane-1-carboxylic acid synthase were significantly suppressed in OA-treated Jujube fruit.

40.4 Metabolomics of Fruit Crops

The constituents of the metabolome reflect the cellular processes that control the biochemical phenotype of the cell, tissue, or whole organism. Metabolomics, which denotes the study of all metabolites produced in an organism, can be treated as the ultimate level of postgenomic analysis because it can reveal modulation in metabolite fluxes controlled by changes within gene expression measured using transcriptomics and/or by analyzing the proteome that elucidates posttranslational control over enzyme activity. Metabolic changes are a major feature of plant genetic modification during plant development as well as biotic and abiotic stress [33, 34]. Therefore, measurements of intracellular metabolites, whether qualitative or quantitative, can reveal the biochemical status of an organism and in turn can be used to monitor stress response [35]. Metabolomics could contribute significantly to the study of stress biology in plants and other organisms by identifying different compounds, such as the by-products of stress metabolism, stress signal transduction molecules, or molecules that are part of the acclimation response of plants.

Metabolomics is complicated by the huge diversity of metabolites in any given species especially in the case of plants that encompass approximately 100 000–200 000 metabolites [36]. Though a number of metabolomic approaches are being used to characterize metabolome of organisms, at present it is impossible to quantitate all the metabolites within a cell, tissue, or organism of any kingdom in any system. This could be due to the fact that no single extraction technique or analytical instrument can isolate and detect every metabolite within a biological sample [37, 38]. It is assumed that a comprehensive plant metabolic profile should include a minimum of carbohydrates, amino acids, organic acids, lipids/fatty acids, vitamins, and various other compound classes such as phenylpropanoids, terpenoids, alkaloids, and glucosinolates along with secondary metabolites that vary according to the species under study [37]. A combination of several different analytical methods, encompassing different separation methods such as gas chromatography (GC) or liquid chromatography and other analytical instruments are required. Mass spectrometry is most widely used because of its high sensitivity and propensity for generating structural information. Nuclear magnetic resonance (NMR) is also a powerful technology because of its reproducibility and robust quantification ability, despite the fact that it has low sensitivity compared to MS. Major approaches used in plant metabolomics research include metabolic fingerprinting, metabolite profiling, and targeted analysis [38, 39]. Metabolomics data handling, analysis, and mining and its integration with other omics platforms have been dramatically improved in recent years because of the development of an array of publicly available bioinformatics tools.

Alterations in the metabolome of a wide number of species in response to a broad range of stresses have been analyzed [40–44]. However, most of these studies have been carried out on nonfruit crops. In fruit crops, very few studies have been carried out for metabolite profiling for the purpose of breeding and stress response. Schauer *et al*. [45] carried out metabolic profiling of leaves and fruit of wild species tomato to survey genetic variation in *Solanum lycopersicum*. Subsequently, metabolite profiling was applied for identification of pericarp metabolite QTL [39] using a set of well-characterized tomato introgression lines [46]. This study resulted in the identification of over 880 metabolite QTL that were stable over two independent harvests, thus highlighting the power of metabolite profiling as a tool for dissecting the genetic basis of metabolism. The same tomato introgression line population was also recently phenotyped at the level of fruit volatiles, which are believed to be key determinants of taste, resulting in the identification of 25 QTL for 23 compounds [47]. Moreover, a detailed analysis of one of the introgression lines (IL8–2-1), which contained massive increases in the volatiles phenylacetaldehyde and phenylethanol, enabled the elucidation of the biosynthesis pathway of phenylethanol [47]. Tikunov *et al*. [48] used nontargeted metabolite profiling using comparative multivariate analysis of a set of 94 contrasting tomato (*Lycopersicon esculentum* Mill.) genotypes covering the variation in the germplasm of commercial tomato varieties. The analysis was based on the profiles of all volatiles that could be detected by the analytical method SPME-GC-MS, which revealed 322 different compounds in the entire genotype set. This covers approximately 80% of the 400 tomato volatile compounds, which have been detected in tomato fruit using different analytical methods. Mintz-Oron *et al*. [49] studied gene expression and metabolism in tomato fruit surface tissues, cuticle, which plays a key role in the survival of plants by serving as the interface between plants and their biotic and abiotic environment. The study revealed unique sets of genes and metabolic pathways consisting of a diverse range of metabolites that are active in the peel at various stages of fruit development and may play an important role in stress response.

A metabolomics approach combining (1)H NMR and gas chromatography–electrospray ionization–time-of-flight mass spectrometry (GC-EI-TOFMS) profiling was employed to characterize melon (*Cucumis melo* L.) fruit [50]. Data analyses revealed several gradients of metabolites in fruit flesh that can be related to differences in metabolism and indicated the suitability of multiblock HPCA for correlation of data from two (or potentially more) metabolomics platforms. The same group also studied metabolic acclimation of melon to hypoxia [51]. Direct (1)H NMR profiling of juice of fruit flesh collected from different locations revealed several gradients of metabolites, for example, sucrose, alanine, valine, GABA, and ethanol, with increase in concentrations from the periphery to the center of the fruit. GC-MS profiling of ground samples revealed gradients for metabolites not detected using (1)H NMR, including pyruvic and fumaric acids. The quantification of adenine nucleotides highlighted a strong decrease in both ATP and ADP ratios and the adenylate energy charge from the periphery to the center of the fruit. These concentration patterns are consistent with an increase in ethanol fermentation due to oxygen limitation and were confirmed by observed changes in alanine and GABA concentrations, as well as other markers of hypoxia in plants.

Global metabolic profiling of "Granny Smith" apple peel was employed for evaluating metabolomic alterations resulting from prestorage UV-white light irradiation [52]. Apples were bagged midseason to restrict sunlight, harvested at the preclimacteric stage prior to bag removal, and treated with fluorescent UV-white light for different time points. Analysis of methanolic extracts from peel samples collected immediately after irradiation or following cold storage were evaluated using GC-MS and LC-UV/vis-MS, respectively. The profile including more than 200 components, 78 of which were identified, revealed changes in the metabolome provoked by UV-white light irradiation and cold storage. Analyses of individual components selected using principal component analysis (PCA) models showed distinct temporal changes, before and after cold storage, related to prestorage irradiation in a diverse set of primary and secondary metabolic pathways. The results demonstrate metabolic pathways associated with ethylene synthesis, acid metabolism, flavonoid pigment synthesis, and fruit texture are altered by prestorage irradiation, and many of the alterations are detectable after 6 months of cold storage in air.

Metabolic fingerprinting of salt stress in tomato was used to identify metabolic changes in fruits under salinity stress [53]. The authors studied two tomato varieties subjected to salinity stress. Whole fruit flesh extracts were fingerprinted using FT-IR spectroscopy. Metabolic fingerprints were analyzed using unsupervised (PCA) and supervised (DFA) algorithms. PCA was not able to discriminate between control and salt-treated groups in any variety, while DFA was able to classify control and salt-treated groups in both varieties [53]. The authors also employed a GA to identify the regions within the FT-IR spectrum that are important for classification. These regions corresponded to saturated and unsaturated nitrile compounds, cyanide-containing compounds, and other nitrogen-containing compounds. In a recent study, it has been shown that salinity stress improves fruit quality of tomato by increasing assimilable metabolism through accumulation of sucrose, citrate, malate, and glutamate in red fruit [54]. To elucidate the mechanisms underlying this phenomenon, Yin *et al.*, [55] studied transport of carbohydrates into tomato fruits and the regulation of starch synthesis during fruit development in plants exposed to high levels of salinity stress. Growth with 160 mM NaCl doubled starch accumulation in tomato fruits compared to control plants during the early stages of development, and soluble sugars increased as the fruit matured. Tracer analysis confirmed that elevated carbohydrate accumulation in fruits exposed to salinity stress was confined to the early development stages and did not occur after ripening. Salinity stress also upregulated sucrose transporter expression in source leaves and increased activity of ADP-glucose pyrophosphorylase (AGPase) in fruits during early developmental stages. The results indicated that salinity stress enhanced carbohydrate accumulation in the form of starch during the early fruit development and is responsible for the increased soluble sugars in ripe fruit.

An integrated study of the early and late changes in transcript and metabolite profiles revealed differences in the dynamics of grapevine response to water and salinity stress [56] and also exhibited differences in molecular response to water deficit and salinity. GC-MS profiling and anion-exchange chromatography with UV

detection revealed that concentration of glucose, malate, and proline is higher in water deficit-treated plants than in salt-stressed plants. These differences in metabolite levels were correlated with differences in transcript levels of many genes involved in energy metabolism and nitrogen assimilation, suggesting a higher demand in water-deficit-treated plants to adjust osmotically, detoxify ROS, and cope with photoinhibition than in salt-stressed plants [56].

All omic-related studies have similar objective of either identifying specific QTL for a set of characters and use it for generating suitable hybrid or identifying a gene/transcription factor that can control/exhibit desired character. In the following section, we have tried to review some of the fruit transgenics that have been generated out of the genes shown to be involved in abiotic stress.

40.5 Stress-Related Fruit Transgenics

Various omic approaches have paved the way for identification of target genes/molecules that are associated with abiotic stress in fruit plants. In an effort to genetically engineer fruit crops for enhanced abiotic stress tolerance, several genes have been used. Because of the relative ease of tomato transformation, most of the studies have been carried out on tomato. Various plants such as *Arabidopsis*, rice, and so on have been used as the source of genes. *Arabidopsis CBF1* gene encoding a drought responsive transcription factor was used to generate transgenic tomato plants that showed tolerance to cold stress, salinity, and drought [58]. However, the use of the CaMV35S promoter for driving expression of the *CBF1* gene caused retarded growth. When the CaMV35 S promoter was replaced by the ABA responsive *HAV22* gene promoter, the transgenic tomato plants showed normal growth but enhanced tolerance to the stresses such as salinity, cold, and drought. Many reports have shown that drought tolerance is associated with increased accumulation of trehalose. The engineering of the trehalose-6-phosphate synthase gene from yeast into tomato under the 35S promoter to increase levels of trehalose indeed led to tolerance to water stress in tomato [58], although there were several developmental changes in the plant morphology. Expression of the *Capsicum anuum PIF1* gene encoding the pathogenesis-induced factor in tomato caused upregulation of several genes from different metabolic pathways and stress responses as deduced by microarray that included genes involved in cold stress. Transgenic plants were tolerant not only to *Pseudomonas syringae* pv. tomato DC 3000 but also to cold stress [59]. Expression of the rice Myb transcription factor gene *OsMYB4* in tomato led to tolerance to drought stress although not to cold stress unlike in rice [60]. The expression of the same gene in apple led to plants being tolerant to both drought and cold stresses. The stress response was associated with higher levels of osmolytes such as glucose, fructose, sucrose, and proline under unstressed conditions as well as during early stages of cold exposure [61]. Transgenic plants, however, were shorter in height compared to control plants. Recently, a detailed insight was obtained into the functioning of the ABA-induced Myb1 gene in tomato, *SlAIM1*, in abiotic stress

(salinity) and oxidative stress, and responses to ABA. RNAi plants with lower levels of *SlAIM1* showed increased susceptibility to salt and oxidative stress, while ectopic expression of *SlAIM1* led to tolerance to these stresses [62]. The mechanism underlying these responses appeared to be through changes in sensitivity to ABA and regulation of ion fluxes such that higher expression levels of the gene caused decreased accumulation of Na^+, while downregulation of the gene caused increased accumulation of Na^+ in tissues. Overexpression of another transcription factor from capsicum CaKR1 (encoding an ankyrin repeat Zn finger domain protein) in transgenic tomato generated plants that showed enhanced tolerance to salinity and oxidative stress through increased activity of *LeSOD2* and *LeAPX2* and *LeAPX3*. Salt tolerance in tomato was engineered by expressing the *Arabidopsis* AtNHX1 gene that encodes a vacuolar Na^+/H^+ antiporter [63]. Tomato plants expressing the *Arabidopsis* AtIpk2 beta gene, encoding an inositol polyphosphate 6-/3-kinase, showed tolerance to cold, drought, and oxidative stress [64]. Other stress-associated compounds such as glycine-betaine have also been enhanced by engineering tomato plants with plastid-directed expression of choline oxidase genes. Transgenic plants showed higher levels of tolerance to oxidative stress [65]. In an interesting study, Orsini *et al.* [66] recently showed that constitutive expression of prosystemin, a wound-inducible polypeptide, decreased stomatal conductance and reduced growth under normal growth conditions, but under conditions of high salt, plants maintained higher stomatal conductance and were tolerant to salt. This indicated that activation of wound responses could counter salt stress. Another gene encoding the *Arabidopsis* tryptophan synthase beta1 (AtTSB1) conferred tolerance to Cd stress in transgenic tomato plants through higher levels of tryptophan [67]. Other genes, such as wheat vacuolar pyrophosphatase gene (TVP1) and the wheat vacuolar sodium antiporter gene (TNHX1), were introduced into tomato and shown to confer salt tolerance [68]. The plants, besides showing higher chlorophyll levels under saline conditions, showed a well-developed root system in TVP1 overexpressing plants.

Apart from tomato, other fruit crops have also been used for manipulation of abiotic stress response. The apple spermidine synthase gene *MaSPD1* was introduced into pear (*Pyrus communis*). The transgenic pear showed enhanced tolerance to salinity, osmotic stress, and increased copper stress. Part of the tolerance was explained by altered polyamine levels, especially higher spermidine levels in transgenic plants, and a greater capacity to withstand oxidative stress as observed by increased SOD and glutathione reductase levels [69, 70]. The increased spermidine levels were responsible for tolerance to increased aluminum concentrations in soil [71] and other heavy metals due to an effect on relieving oxidative stress [72]. An increase in polyamine levels (spermine and spermidine) and tolerance to heat stress due to enhanced antioxidant enzyme activity and reduced lipid peroxidation was also achieved in transgenic tomato by introduction of the yeast SAM decarboxylase gene [73]. In another study, constitutive expression of the heat shock gene *HSP21* in tomato resulted in greater protection to PSII from temperature-dependent oxidative stress. In addition, plants showed earlier accumulation of carotenoids [74].

40.6 Conclusions

The yield potential of all crop plants including fruit plants is greatly affected by the abiotic stress conditions such as drought, water deficit, cold, heat, flooding, and chemicals. These abiotic stresses bring about osmotic stress and/or oxidative stress that disrupt functional and/or structural proteins and membranes. In many cases, it has been observed that wild relatives of the cultivated crops could withstand these stresses and survive. This indicates that these plants acclimatize by altering their physiology through differential expression of genes that results in accumulation of certain proteins and metabolites. In order to identify those genes, proteins, and metabolites, it is important to look into global changes in transcripts, proteins, and metabolite profiles during abiotic stress conditions. Omic approaches offer such kind of analyses where entire transcriptome, proteome, and metabolome can be studied under one set of conditions. Several high-end equipments are available that either singly or in combination can give accurate measurements of transcripts, proteins, and metabolites. Abiotic stress and fruit yield and quality are relatively newer areas where not much study has been done. Though whole-genome sequences are available for papaya, grape, and tomato, and for other fruits such as banana, apple, and citrus will be available shortly, most studies have so far been done with respect to identifying genes that are expressed differentially during different kinds of abiotic stresses. Only in some cases, global transcriptome, proteome, or metabolome have been analyzed. Transgenic tomato, apple, and pear plants have been raised with one or the other genes that conferred resistant to abiotic stresses. The whole-genome sequencing of various fruit and other plants has indicated that 20–40% of genes account for no known functions. We make an optimistic assumption that at least some of these genes are involved in stress tolerance and manipulation of these genes may lead to transgenics resistant to abiotic stress.

References

1 Boyer, J.S. (1982) *Science*, **218**, 443–448.
2 Wang, W. and Altman, A. (2003) *Planta*, **218**, 1–14.
3 Mittler, R. and Blumwald, E. (2010) *ARPB*, **61**, 13.1–13.20.
4 Brouder, S.M. and Volenec, J.J. (2008) *Physiol. Plant.*, **133**, 705–724.
5 Horan, K., Jang, C., and Bailey-Serres, J. *et al.* (2008). *Plant Physiol.*, **147**, 41–57.
6 Gollery, M., Harper, J., Cushman, J. *et al.* (2006) *Genome Biol.*, **7**, R57.
7 Romero, P., Navarro, J.M., Pérez-Pérez, J. *et al.* (2006) *Tree Physiol.*, **26**, 1537–1548.
8 Keutgen, A.J. and Pawelzik, E. (2007) *J. Agri. Food Chem.*, **55**, 4066–4072.
9 Demetriou, K., Kapazoglou, A., Tondelli, A. *et al.* (2009) *Physiol. Plant.*, **136**, 358–368.
10 Gupta, V., Mathur, S., Solanke, A.U. *et al.* (2009) *Crit. Rev. Biotechnol.*, **29**, 152–181.
11 Islam, M.S. and Wang, M.H. (2009) *BMB Rep.*, **42**, 611–616.
12 Wang, H., Huang, Z.J., Chen, Q. *et al.* (2004) *Plant Mol. Biol.*, **55**, 183–192.
13 Zhang, H.W., Huang, Z.J., Xie, B.Y. *et al.* (2004) *Planta*, **220**, 262–270.
14 Zhang, X., Fowler, S.G., Cheng, H.M. *et al.* (2004) *Plant J.*, **39**, 905–919.
15 Yanez, M., Caceres, S., Orellana, S. *et al.* (2009) *Plant Cell Rep.*, **28**, 1497–1507.

16 Weiss, J. and Egea-Cortines, M. (2009) *J. Appl. Genet.*, **50**, 311–319.
17 Maul, P., McCollum, G.T., Popp, M. *et al.* (2008) *Plant Cell Environ.*, **31**, 752–768.
18 Wisniewski, M., Bassett, C., Norelli, J. *et al.* (2008) *Physiol. Plant.*, **133**, 298–317.
19 Davey, M.W., Graham, N.S., Vanholme, B. *et al.* (2009) *BMC Genomics*, **10**. doi: 10.1186/1471-2164-10-436
20 Castellarin, S.D., Pfeiffer, A., Sivilotti, P. *et al.* (2007) *Plant Cell Environ.*, **30**, 1381–1399.
21 Rocco, M., D'Ambrosio, C., Arena, S. *et al.* (2006) *Proteomics*, **6**, 3781–3791.
22 Faurobert, M., Mihr, C., Bertin, N. *et al.* (2007) *Plant Physiol.*, **143**, 1327–1346.
23 Giribaldi, M., Perugini, I., Sauvage, F.X., and Schubert, A. (2007) *Proteomics*, **7**, 3154–3170.
24 Neilson, K.A., Gammulla, C.G., Mirzaei, M. *et al.* (2009) *Proteomics*, **10**, 828–845.
25 Ahsan, N., Lee, D.-G., Lee, S.-H. *et al.* (2007) *Physiol. Plant.*, **131**, 555–570.
26 Zhou, S., Sauvé, R., and Thannhauser, T.W. (2009) *J. Exp. Bot.*, **60**, 1849–1857.
27 Hattrup, E., Neilson, K.A., Breci, L., and Haynes, P.A. (2007) *J. Agric. Food Chem.*, **55**, 8310–8318.
28 Lücker, J., Laszczak, M., Smith, D., and Lund, S.T. (2009) *BMC Genomics*, **10**. doi: 10.1186/1471-2164-10-50
29 Grimplet, J., Wheatley, M.D., Jouira, H.B. *et al.* (2009) *Proteomics*, **9**, 2503–2528.
30 Lara, M.V., Borsani, J., Budde, C.O. *et al.* (2009) *J. Exp. Bot.*, **60**, 4315–4333.
31 Qin, G., Wang, Q., Liu, J. *et al.* (2009) *Proteomics*, **9**, 4241–4253.
32 Wang, Q., Lai, T., Qin, G., and Tian, S. (2009) *Plant Cell Physiol.*, **50**, 230–242.
33 Schauer, N. and Fernie, A.R. (2006) *Trends Plant Sci.*, **11**, 508–516.
34 Bräutigam, K., Dietzel, L., Kleine, T. *et al.* (2009) *Plant Cell*, **21**, 2715–2732.
35 Fiehn, O., Kopka, J., Dörmann, P. *et al.* (2000) *Nat. Biotechnol.*, **18**, 1157–1161.
36 Oksman-Caldentey, K.-M. and Inzé, D. (2004) *Trends Plant Sci.*, **9**, 433–440.
37 Sumner, L.W., Mendes, P., and Dixon, R.A. (2003) *Phytochemistry*, **62**, 817–836.
38 Allwood, J.W., Ellis, D.I., and Goodacre, R. (2008) *Physiol. Plant.*, **132**, 117–135.
39 Schauer, N., Semel, Y., Roessner, U. *et al.* (2006) *Nat. Biotechol.*, **24**, 447–454.
40 Kaplan, F., Kopka, J., Haskell, D.W. *et al.* (2004) *Plant. Physiol.*, **136**, 4159–4168.
41 Hamzehzarghani, H., Kushalappa, AC., Dion, Y. *et al.* (2005) *Physiol. Mol. Plant Pathol.*, **66**, 119–133.
42 Nikiforova, VJ., Kopka, J., Tolstikov, V. *et al.* (2005) *Plant. Physiol.*, **138**, 304–318.
43 Hirai, M.Y. and Saito, K. (2004) *J. Exp. Bot.*, **55**, 1871–1879.
44 Holmstrom, K.O., Somersalo, S., Mandal, A. *et al.* (2000) *J. Exp. Bot.*, **51**, 177–185.
45 Schauer, N., Zamir, D., and Fernie, A.R. (2005) *J. Exp. Bot.*, **56**, 297–307.
46 Eshed, Y. and Zamir, D. (1994) *Euphytica*, **79**, 175–179.
47 Tieman, D., Taylor, M., Schauer, N. *et al.* (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 8287–8291.
48 Tikunov, Y., Lommen, A., de Vos, C.H.R. *et al.* (2005) *Plant Physiol.*, **139**, 1125–1137.
49 Mintz-Oron, S., Mandel, T., Rogachev, I. *et al.* (2008) *Plant Physiol.*, **147**, 823–851.
50 Biais, B., Allwood, J.W., Deborde, C. *et al.* (2009) *Anal. Chem.*, **81**, 2884–2894.
51 Biais, B., Beauvoit, B., Allwood, J.W. *et al.* (2010) *J. Plant Physiol.*, **167**, 242–245.
52 Rudell, D.R., Mattheis, J.P., and Curry, E.A. (2008) *J. Agric. Food Chem.*, **56**, 1138–1147.
53 He, J., Broadhursta, D., Goodacrea, R., and Smith, A.R. (2003) *Phytochemistry*, **62**, 919–928.
54 Saito, T., Matsukura, C., Ban, Y. *et al.* (2008) *J. Japanese Soc. Hort. Sci.*, **77**, 61–68.
55 Yin, Y.G., Kobayashi, Y., Sanuki, A. *et al.* (2010) *J. Exp. Bot.*, **61**, 563–574.
56 Cramer, G.R., Ergül, A., Grimplet, J. *et al.* (2007) *Funct. Integr. Genomics*, **7**, 111–134.
57 Lee, J.T., Prasad, V., Yang, P.T. *et al.* (2003) *Plant Cell Environ.*, **26**, 1181–1190.
58 Cortina, C. and Culianez-Macia, F.A. (2005) *Plant Sci.*, **169**, 75–82.
59 Seong, E.S., Baek, K.H., Oh, S.K. *et al.* (2007) *Physiol. Plant.*, **129**, 555–566.
60 Vannini, C., Campa, M., Iriti, M. *et al.* (2007) *Plant Sci.*, **173**, 231–239.
61 Pasquali, G., Biricolti, S., Locatelli, F. *et al.* (2008) *Plant Cell Rep.*, **27**, 1677–1686.
62 Abu Qamar, S., Luo, H.L., Laluk, K. *et al.* (2009) *Plant J.*, **58**, 347–360.
63 Zhang, H.X. and Blumwald, E. (2001) *Nat. Biotechnol.*, **19**, 765–768.
64 Zhang, Y., Liu, H., Li, B. *et al.* (2009) *Transgenic Res.*, **18**, 607–619.

65 Park, E.J., Jeknic, Z., Pino, M.T. *et al.* (2007) *Plant Cell Environ.*, **30**, 994–1005.

66 Orsini, F., Cascone, P., and De Pascale, S. (2010) *Physiol. Plant.*, **138**, 10–21.

67 Sanjaya, M., Hsiao, P.Y., Su, R.C. *et al.* (2008) *Plant Cell Environ.*, **31**, 1074–1085.

68 Khoudi, H., Nouri-Khemakhem, A., Gouiaa, S., and Masmoudi, K. (2009) *Afr. J. Biotechnol.*, **8**, 6068–6076.

69 Wen, X.-P., Pang, X.-M., Matsuda, N. *et al.* (2008) *Transgenic Res.*, **17**, 251–263.

70 He, L., Ban, Y., Inoue, H. *et al.* (2008) *Phytochemistry*, **69**, 2133–2141.

71 Wen, X.-P., Ban, Y., Inoue, H. *et al.* (2009) *Environ. Exp. Botany*, **66**, 471–478.

72 Wen, X.-P., Ban, Y., Inoue, H. *et al.* (2010) *Transgenic Res.*, **19**, 91–103.

73 Cheng, L., Zou, Y.J., Ding, S.L. *et al.* (2009) *J. Integr. Plant Biol.*, **51**, 489–499.

74 Neta-Sharir, I., Isaacson, T., Lurie, S., and Weiss, D. (2005) *Plant Cell*, **17**, 1829–1838.

41
Cassava Genetic Improvement: Omics Approaches for Facing Global Challenges

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Cassava is a staple food crop that ensures food security and is a source of income generation for poor farmers in the tropics of many Asian and African countries that are highly prone to harsh and unfavorable environments especially in the context of climate changes. Improving tolerance to abiotic stresses such as drought in this crop is a big challenge as it has substantial tolerance to drought compared to other crops. There has been little investment in employing advanced breeding technology for genetic improvement of cassava, though we have now an increased access to emerging advanced tools in genomics, transcriptomics, phenomics, proteomics, and metabolomics for that can enhance our capacity to understand stress responses and tolerance in plants.

In this chapter, we have reviewed challenges and opportunities for improving productivity of cassava by integrating omics with conventional breeding. The chapter highlights efforts and vision of International Center for Tropical Agriculture (CIAT; http://www.ciat.cgiar.org/) toward a comprehensive evaluation of germplasm, establishment of the transformation and phenotyping platform, and development of genomic tools for deriving the requisite information for developing effective strategies for molecular breeding for high-yield and high-value cassava under stress conditions. The chapter also deals with possible strategies to accelerate development of elite cassava cultivars by integration of the advanced technologies.

41.1
Introduction

Cassava is an important tropical crop both for food security and for income generation for poor farmers in many Asian and African countries, where its production is more than 240 million ton a year [1]. This crop serves as the primary food source for more than 750 million people across the world. Cassava is well adapted to semiarid conditions and it needs adequate soil moisture mainly during

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planting. After establishment, it can withstand several months of drought. Generally, it is not irrigated, but in some areas responds markedly to irrigation [2]. However, severe limitation of soil moisture can bring down the cassava yield significantly [3–5]. So, the task of improving the productivity of this drought-tolerant crop under soil moisture stress environments is more challenging than for other crops.

In several investigations supported by the Generation Challenge Programme (GCP: http://www.generationcp.org/), in Brazil, Colombia, Tanzania and Ghana, contrasting cassava varieties have been screened and evaluated under drought environments and physiological traits associated with drought tolerance have been identified [6–8]. Among these, there are traits related to developmental regulation that permits the most drought-tolerant lines to optimally partition resources for storage root initiation and maintenance during drought. However, despite substantial accumulation of basic knowledge of cellular and molecular level, there is no significant progress toward development of drought-tolerant varieties mainly because of limited success in dissecting the complex mechanisms underlying tolerance to drought.

Recent remarkable innovations in omics-based research such as phenomics, genomics, transcriptomics, and application development can provide us new opportunities to resolve the complexities associated with drought tolerance. Furthermore, these advances in science can also provide us crucial resources to promote research both in crop plants and in model plants [9–12]. All kinds of "omics," arrays, and high-throughput technologies can now make it possible to carry out genetic and genomic analysis for the investigation of gene function associated with phenotypic changes on a larger scale to accelerate crop breeding.

In this chapter, we have reviewed the recent efforts to improve cassava especially for drought tolerance and have presented perspectives for integrating the knowledge that has been accumulated through the traditional research and the current advanced technologies to improve cassava genotypes with an efficient and targeted approach.

41.2
What Makes the Cassava More Tolerant to Drought?

Cassava tolerates prolonged drought that often exceeds 5 months. This has been attributed to partial closure of stomata, deep rooting systems, and small leaf canopy. These traits make cassava a desirable and adaptable crop, as a source for food and feed, in the hot and dry tropics that are highly prone to predicted adverse global climate changes [13, 14].

Recent review by El-Sharkawy [8] reveals several attempts made at understanding the mechanisms involved in the drought tolerance in cassava and its possible application in breeding programs for enhancing its productivity [8]. Some of the traits relevant to drought tolerance have been listed in Table 41.1. The challenge of further improving cassava for the productivity of harvestable roots now largely lies in

Table 41.1 Plant traits contributing to drought tolerance in cassava.

Trait	Contribution to drought and productivity	Conditions under which it is useful
Photosynthesis	High rate of photosynthesis under favorable conditions and maintenance even under prolonged drought conditions and recovery	General
Tight stomatal control	Protection from both low soil moisture and high evaporative demand	Hot environment with no soil moisture in deeper soil profile
Plant vigor	High vegetative vigor enables plants to recover from drought	Intermediate drought conditions
C3–C4 intermediate mechanisms	These characteristics, collectively, underpinned the high photosynthetic rate (P_n) in normal air ($P_n > 40\,\mu mol\,CO_2\,m^{-2}\,s^{-1}$) in high irradiances (>1800 μmol $m^{-2}\,s^{-1}$ of photosynthetic active radiation), high leaf temperature from 30 to 40 °C, and in high atmospheric humidities observed in cassava grown in favorable environments [6, 15]	Environments where high evaporative demand exist
Shoot morphological adjustments	During prolonged water deficits, cassava reduces its canopy by shedding older leaves and forming smaller new leaves leading to less light interception	Intermediate drought
Leaf area index	Optimum leaf area for longer period during the drought and after recovery	Normal and intermediate drought conditions
Deep roots	Cassava slowly extracts soil moisture from deeper layers of soil	Drought environments with moisture stored in deeper layers of soil
Predawn leaf water potential	Relative to unstressed plants	Intermediate drought

our efforts to phenotype known and unknown traits of drought tolerance. This can be accomplished through optimized and novel phenotyping techniques that can generate highly reliable data on plant response and stress environments for complementing genomic information.

41.3 Phenomics for Trait Discovery

Breeders' eye approach with large dependence on empirical selection for yield, disease, and pest resistance has significantly contributed to historical jump in food production during the Green Revolution in the past. However, scope for continuing this approach is gradually diminishing as evident from the recent yield plateau in many of the staple foods such as rice, wheat, and maize. The rapidly developing genomic knowledge and genotyping capacity can offer us opportunities to improve crop varieties in a much more targeted way to grow them in harsh environments, for more productivity with fewer inputs. Genomics and transcriptomics in combination with bioinformatics can offer great opportunity to generate knowledge about the function of the genome, unraveling genomic pathways, but more importantly, to identify useful diversity, genes, and alleles and transfer these rapidly into adapted varieties. However, these new approaches and tools need to be sufficiently complemented by screening protocols that can facilitate generation of high-quality data on plant phenotypes under given stress environment. This necessarily should include understanding and management of stress environment and rapid and noninvasive procedures to measure response of the large number of genetically diverse genotypes created through conventional breeding or genetic engineering approaches. These efforts with new terminology, "phenomics," have now become the focal point of much of the agricultural research aiming to get benefits from advances in genomics.

One of the major tasks in phenomics is the determination of plant responses to stress in well-defined environment that can clearly reveal the genetic variation. This will lead to trait discovery that can be eventually associated with gene(s) through quantitative trait locus (QTL) cloning or/and transgenic approach. In addition, the phenomics should focus on inherent gaps in understanding the magnitude of drought stress in terms of duration of soil moisture stress and level of soil moisture during a specific growth period in crops such as cassava.

In a classical example of a field evaluation, it was shown that the reduction in root yield in response to 120 days of drought imposed 60 days after planting could reduce the yield marginally compared to the control in which soil moisture level was maintained at field capacity. In this experiment, some of the genotypes had more grain yield compared to control even after exposure to the drought [8]. Furthermore, compared to the control, water stress across all varieties caused a reduction in total biomass by 12%, no change in fresh root yields, a reduction in dried root yields by 3.4%, a reduction in dry matter contents by 3.3%, and an increase in the harvest index by 10%. Without any doubt, these data clearly demonstrate cassava's ability to tolerate prolonged water stress when it is induced gradually at an early stage of growth and also its ability to recover from drought. However, the magnitude of drought stress, the duration, and critical phase of growth in such experiments need a review as possibly the soil moisture deficit was not sufficient enough to cause substantial impact on productivity of cassava genotypes, to elucidate genetic variation in the response, which is fundamental to further genetic improvement. Alternatively, control plants did not have sufficient scope to express their yield potential. Hence, we suggest that

the challenge of further improving the productivity of cassava under moisture stress should consider a reoriented phenotyping protocol.

1) To dissect traits contributing to components of crop productivity under soil moisture deficit [16].
2) Cassava root yield = water uptake × water use efficiency × partitioning to roots that can be harvested.
3) To impose drought at critical stage of growth that determines productivity of crop.
4) The time when number of productive roots determined.
5) Root bulking.
6) To reduce the influence of phenological variation in understanding genetic variation in drought response, through staggered sowing.
7) To differentiate genetic variation in recovery from drought stress.
8) To understand the yield gap in the target environment that needs to be covered by genetic improvement of traits associated with high root yield. The traits listed in Table 41.2 may be considered depending on the nature of drought stress.

41.4
How Cassava Genomics Tool will help Develop Drought-Tolerant Variety

In 2007, CIAT and RIKEN initiated an international collaborative research project and since 2009, a 3-year cassava research project, between RIKEN (Japan, PI: Dr. Motoaki Seki), CIAT (Colombia, PI: Dr. Manabu Ishitani), and Mahidol University (Thailand, PI: Dr. Jarunya Narangajavana), which is supported by the Japanese government, has started to develop an integrated cassava genome analysis platform for the global cassava research community and to apply it to molecular breeding for cassava improvement. In the collaborative work, the following cassava genomics tools are being developed: (1) large-scale collection of full-length cDNAs ([17], unpublished data), (2) cassava oligoarray analysis platform containing more than 30 000 genes, and (3) cassava database of international standard. For the collection of full-length cDNA clones, we have selected three genetically diverse varieties, namely, KU50 (*Manihot esculenta* Crantz), MEcu-72 (*M. esculenta* Crantz), and MPer 417-003 (*M. esculenta* peruviana). KU50 is a commercial cultivar developed in Thailand, which is extensively grown in Southeast Asia. MEcu-72 is one of the naturally occurring wild landraces in Ecuador reported to be resistant to whiteflies by CIAT [18]. MPer 417-003 is a wild species from Brazil, which is probably involved in the evolution of cassava cultivar, *M. esculenta* Crantz. The MPer 417-003 has strong resistance to insects such as mealybugs, mites, and whiteflies [19]. One of the objectives to choose these varieties is to understand common mechanisms of drought tolerance in cassava and use this knowledge for the improvement of other crops mainly because cassava has some features that allow it to cope with stress better than other crops, for example, high stomatal sensitivity to environmental humidity [20] and quick recovery after stress [21]. Another objective is to identify novel traits and superior genes using genetic diversity existing in cultivated and wild species of cassava. So far, we have obtained 19 968, 29 952, and 19 968 end sequences of full-

Table 41.2 Choosing right trait for right drought environment while screening the germplasm. Asterisks indicate the level of the relevance between trait and drought tolerance (*=low, **=middle and ***=high)..

Major trait	Associate traits	Trait relevance to drought at different growth stages		
		Early drought	Mid season	Late drought
Water uptake	Deep root		***	***
	Temporal root architecture		***	***
	Cool canopy	**	**	
Water use efficiency	Partial closure of stomata	***	***	
	High photosynthesis	***	***	
	Optimum leaf area index after recovery	***	***	*
	Early growth vigor	*	***	***
Better partitioning to roots	Early bulking	*	*	***
	Stems with more storage capacity for nonstructural carbohydrates	*	**	***
	Efficient translocation of nonstructural carbohydrates to roots		**	***

length cDNA clones from KU50, MEcu-72, and MPer 417-003, respectively and some of the sequences are not present on Phytozome Web site (www.phytozome.net/cassava), the cassava genome database. Using a set of unique genes from the expressed sequence tag (EST) collections, an oligoarray analysis platform is being developed with more than 30 000 genes. These genomic and transcriptomic tools enable us to dissect traits of importance for cassava improvement in a comprehensive way. We believe that the platform could be applied for any cassava variety including wild cassava species and will be useful to cassava research community globally.

In 2009, the first annotated draft cassava genome sequence became available at the Phytozome Web site (www.phytozome.net/cassava). The genome sequencing project has been initiated through the DOE-JGI Community Sequencing Program (CSP) by a 14-member consortium including DanForth Plant Center and CIAT. Furthermore, the University of Arizona has started a new project funded by the Bill & Melinda Gates Foundation in collaboration with DOE-JGI, 454 Life Sciences, and the University of Maryland, Baltimore, to develop a genome variation database that will provide breeding tools such as markers. This variation database with comprehensive genome information from different cassava genotypes will help make breeding schemes more efficient through marker-assisted selection (MAS) (http://www.bio5.org/news/news_release/13-million-grant-ua-bill-melinda-gates-foundation-will-fund-next-phase-cassava-res). The full-length cassava cDNA sequence information derived from different cassava varieties will significantly contribute to cassava genome sequence annotation and help know the source of genetic variation in different cassava species. The high quality and correct annotation of the genome sequence can further accelerate identification of useful genes and promoters associated with traits contributing to drought tolerance in cassava through QTL, transcriptomic analysis (e.g., DNA microarrays), and transgenic approaches. In *Arabidopsis* and rice, molecular responses to drought stress have been intensively analyzed and several key genes involved in the drought stress response and tolerance have been identified [22]. As most of the knowledge that has been obtained so far in *Arabidopsis* and rice can be applied to other plants [23, 24], cassava homologues of the drought stress-related genes in *Arabidopsis* and rice can allow us to look at genetic difference in terms of gene and gene duplication between *Arabidopsis*, rice, and cassava in terms of drought stress response and its tolerance among the plant species.

41.5
Gene Discovery for Drought Tolerance in Cassava

QTL mapping becomes a standard procedure in quantitative genetics [25] and recent technical progress in the area of molecular biology and genomics have allowed us to identify QTL gene. There are two major strategies for the QTL cloning: one is through positional cloning and another is through association mapping. Because of a long growth cycle and complex genetic nature of cassava, gene discovery through QTL cloning was not an easy task in the past and this will drastically change once all the genomic sequences and genomics tools become available through current global

efforts as described above. The tools will help develop fine genetic mapping, physical mapping, or markers, which are strongly associated with trait of interest for cassava improvement [26–28]. In addition, plant comparative genomics and transcriptomics approach will largely benefit from research advances described above to discover key genes associated with traits of interest [29–32]. Especially, sequence homology or similarity can serve as a strong evidence for detecting functional elements in genomic sequences. Here, we give an example to identify cassava genes, which may have molecular and physiological functions similar to those of genes in other plants.

It was recently reported that transgenic cassava expressing *IPT* gene, which encodes isopentenyl transferase from *Agrobacterium tumefaciens*, under the control of the *Arabidopsis* promoter of the senescence-induced *SAG12* gene, could trigger a series of metabolic changes such as cytokinin metabolism that increased drought resistance [33]. This study not only could prove the feasibility of delayed leaf senescence of woody cassava but also could reveal mechanisms underlying the control of cytokinin homeostasis in cassava leaves. However, the origin of the IPT gene used in this study was *A. tumefaciens*, a bacterium. Therefore, we carried out homologue search by BLAST program and phylogenetic analysis by NJ method to identify orthologue genes of IPT in cassava, and nine sequences were identified from cassava genome database as shown in Figure 41.1. Generally, *IPT* genes are classified into bacterial type or plant type according to their amino acid sequences [34]. Interestingly, plant-type *IPT* genes in *Arabidopsis* are strongly involved in the cytokinin metabolism in contrast to bacterial-type *IPT* genes [34–36]. As shown in Figures 41.1 and 41.2, all the nine genes identified as cassava *IPT* genes belong to the plant-type IPT group. Furthermore, some cassava EST sequences, which partially encode the plant-type *IPT* genes, are now available. We are trying to isolate full-length cassava cDNAs encoding plant-type *IPT* genes for developing drought-tolerant cassava cultivars using transgenic approach.

It is recognized that traditional breeding is most unlikely to provide all solutions for improving the crop to meet the varying needs of small farmers and commercial production in the tropics [39, 40]. The transgenic technologies can accelerate integration of desired traits into farmer preferred cultivars and landraces, as well as elite breeding lines. Hence, this technique is one of the options to get maximum benefit from major advances occurring in the genomics for improvement of cassava, which is a long-duration crop. Transgenic technologies allow beneficial traits to be transferred from one cassava cultivar to another and from wild relatives to cultivated *Manihot*, circumventing species boundaries and the problems of outcrossing and inbreeding depression, inherent to this vegetatively propagated crop.

The first series of cassava transgenic technologies were reported in 1996 [41–43]. Since then, the transgenic technologies have been improved [44, 45] and utilized to produce genetically transformed cassava expressing traits such as reduced cyanogenic content [46–48], enhanced starch production [49, 50], insect resistance [51], virus resistance [52–56], and herbicide resistance [57, 58]. Recently, it has been reported that transgenic cassava expressing *IPT* gene from *Agrobacterium* increased drought resistance as mentioned in the previous section [33]. The transgenic plants were useful both to investigate drought resistance in cassava and to increase storage

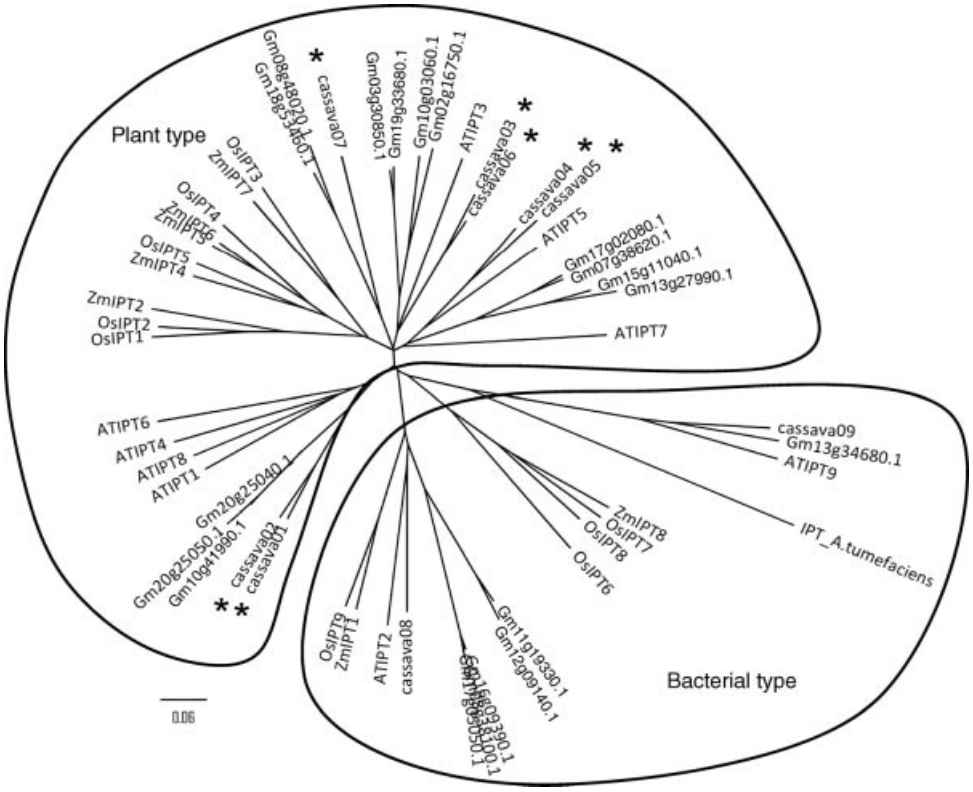

Figure 41.1 Phylogenetic tree of *IPT* genes. The amino acid sequences of the *IPT* genes (9 identified *IPT* homologous genes in *M. esculenta* (cassava), 19 *IPT* homologous genes in *G. max* (Gm) and 9 genes in *A. thaliana* (At) [34], 9 genes in *O. sativa* (Os) [37], and 7 genes in *Z. maiz* (Zm) [38] were aligned by ClustalX2 and the phylogenetic tree was constructed using the NJ method. *IPT* gene in *A. tumefaciens* is a query for the BLAST search. Asterisks show the plant type putative *IPT* genes identified from cassava genome database (phytozome). *IPT* genes are classified into bacteria type or plant type according to their amino acid sequences [34].

root production in semiarid regions and leaf biomass as vegetable and animal feed. Furthermore, Saelim *et al.* [59] have established a marker-free cassava transformation protocol using a cassava cultivar, KU50, which is extensively grown in Southeast Asia. This is the first demonstration of the efficacy of the system in promoting excision of *ipt* marker gene in cassava species, with the consequent rapid production of marker-free transgenic plants [59]. The high efficiency of this system should facilitate pyramiding a number of transgenes by repeated transformation without having to undergo laborious, expensive, and time-consuming processes of sexual crossing and seed production. The generation of marker-free, thus environmentally safe, genetically modified cassava clones should also ease the public concerns regarding the use of transgenic cassava in both food and nonfood industries. Technologies for transformation of plants are in high demand and are being increasingly employed by large seed and agrochemical sectors. Most of the academic research groups lack

infrastructure, logistics and human resources necessary to establish middle to high-throughput transformation pipeline for their own target crop. CIAT has been working in transgenic research on cassava and rice for more than a decade and has scaled up its facilities to include confined fields for field evaluation, and it has been uniquely placed to evaluate the gene technology with a generic permission from National Biosafety Committee of Colombia. The CIAT has now established the Transformation Platform to host all the in-house or collaborative research projects that aim to evaluate transgenic technology for important crops such as rice and cassava. The strategy is to accelerate evaluation of gene technology through transformation and phenotyping platforms by employing scaled up infrastructure, expertise, and knowledge with the ultimate objective of complementing conventional crop improvement technology for cassava.

41.6
Proteomics and Metabolomics Approach in Cassava

Several groups have shown the profiles of protein pattern of a storage root [60], fibrous and tuberous root [61], plant–pathogen interactions [62], and secondary somatic embryogenesis [63] in cassava by using a technology of two-dimensional gel electrophoresis. Recently, Li *et al.* [64] provided a new insight into the proteomic patterns in the somatic embryos, plantlets, and tuberous roots [64]. These investigations have paved the way for a comprehensive, system-wide analysis of cassava. Large-scale proteome data sets are an important resource for a better understanding of protein functions in terms of not only growth and development but also response to environmental changes.

Metabolomics aims to understand metabolic systems based on comprehensive and integrated approaches that take advantage of recent advances in instrumentation to characterize metabolites. Metabolomic approaches can allow us to conduct parallel assessments of multiple metabolites and can facilitate quantitative analysis of particular metabolites with major advantages over chemical-level phenotyping and diagnostic analysis. The plant metabolome represents an enormous chemical diversity due to the complex set of metabolites produced in each plant species [65, 66]. Various sources of information on metabolomics have played a crucial role in metabolome research and its synergistic integration with other omics approaches. Metabolomics information for cassava has not been launched yet, but for other plants, there are Web sites on metabolome resources such as TAIR (http://www.arabidopsis.org/portals/metabolome/index.jsp), the KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html) [67–69], the Plant Metabolic Network (PMN) (http://www.plantcyc.org/), PlantCyc including AraCyc and PoplarCyc (http://www.plantcyc.org:1555/PLANT/server.html) [70], KaPPA-View (http://kpv.kazusa.or.jp/kappa-view/) [71, 72], and PRIMe (http://prime.psc.riken.jp/) [73]. However, the analysis of the ESTs for full-length cDNAs from abiotic and biotic stress-treated cassava tissues mentioned in the previous section has revealed that some of EST sequences encode novel plant genes and they do not belong to any pathways that are already known in plants (unpublished data). It indicates a

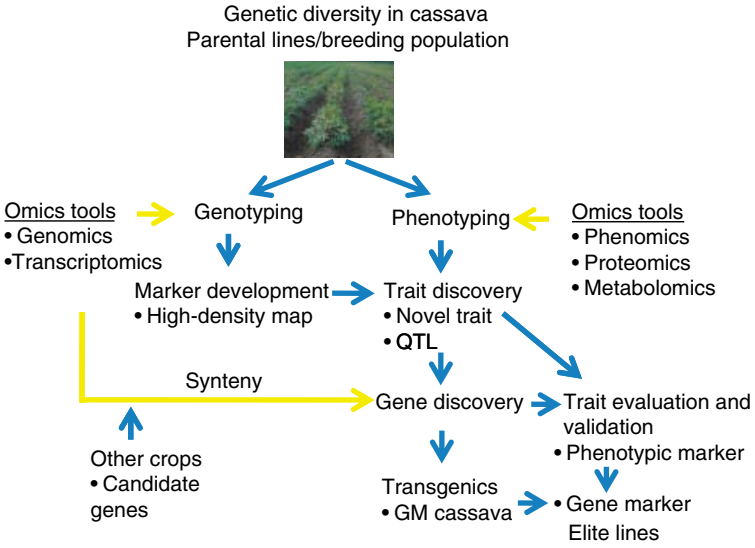

Figure 41.2 Gene discovery for a particular trait is the key to accelerating genetic improvement of cassava; however, genotyping and phenotyping tools are the major prerequisites that can determine the success. Recent advances in genomics can ensure high-density genetic maps, while precision phenotyping is essential for identifying the key traits associated with desired phenotype of plants. This will also determine the precision of association between the trait of interest and the QTL. Omics tools such as genomics and transcriptomics can facilitate both high-quality genotyping and discovery of candidate genes by assessing synteny with other crops. Phenomics, proteomics, and metabolomics can improve efficiency of trait discovery. Thus, the gene discovery will be the product of integration between efficient genotyping and phenotyping. It is necessary to further carry out trait evaluation and identify precise phenotypic marker to carry forward the gene discovery into useful gene marker and to support marker-aided selection in the cassava breeding program.

possibility of the existence of some novel pathways related to stress tolerance in cassava. Therefore, cassava metabolomics, which is a great analytical challenge, can serve as an immensely important tool to design molecular breeding strategies for improvement of the productivity and value of cassava in the near future.

41.7 Future Perspectives

Earlier investigations have often indicated the relationship of some of the physiological traits with root yield of cassava under water deficit environment. The concepts evolved so far need to be validated and transformed into crop improvement tools to ensure precise phenotyping for trait discovery and gene identification. Phenomics platform with tools and techniques that are emerging out of advances in digital imaging and image analysis can significantly help in phenotyping long duration plants such as cassava with reduced cost. These noninvasive rapid methods can greatly complement

research efforts of both the genomic analysis platform and the transformation platform to enhance our understanding about molecular mechanisms of drought tolerance in cassava. However, these technologies and knowledge that can save time and money in developing elite cultivars have not been effectively integrated into cassava breeding programs. This can be partially because of heterozygosity and long growth cycle, which are the major constraints in cassava breeding programs. Furthermore, as shown in Figure 41.2, the key lies in integration of genomics, transcriptomics, metabolomics, proteomics, and other large-scale "omics" data with systems biology approaches, and it can open new avenues for engineering cassava to enhance yields, improve nutritional value, and overcome the problems of postharvest physiological deterioration as well as biotic and abiotic stresses.

Through integrated omics approach, gene markers can be identified rapidly and at low cost in the near future. Furthermore, MAS that focuses on key traits contributing to drought tolerance can become more robust for accelerating genetic improvement of cassava. Since drought tolerance is a highly complex trait, pyramiding multiple sources of genes for drought tolerance into a set of new progenitors can lead to a much desirable impact. CIAT will increasingly adopt technologies evolving from recent developments in single-nucleotide polymorphisms (SNPs) to accelerate the application of MAS strategies in cassava breeding programs. Marker genotyping based on SNP resources are being developed by using next-generation sequencers (NGS) such as 454 DNA sequencer and Illumina genome analyzer. The computational discovery of EST-based SNPs and/or EST-SNP markers is now in progress for numerous species [74–82]. EST data sets for full-length cDNA, which we have obtained (see Section 41.5), will serve as an important resource for discovery of SNPs, especially for locating expressed genes onto a genetic map. This will lead topositional cloning of genes of interest that can be useful as efficient markers for accelerating improvement of cassava for tolerance against abiotic and biotic stresses.

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References

1 FAO. FAOSTAT Statistical Database, Agriculture Data.
2 Vries, C.A., Ferweds, J.D., and Flash, M. (1976) Choice of crops in relation to actual and potential production in tropics. *Neth. J. Agric. Sci.*, **15**, 241–246.
3 de Oliveira, S.L., Macedo, M.M.C., and Porto, M.C.M. (1982) Effect of water deficit on cassava root yield. *Pesqui. Agropecu. Bras.*, **17**, 121–124.
4 Connor, D.J. and Cock, J.H. (1981) Response of cassava to water shortage. II.

Canopy dynamics. *Field Crops Res.*, **4**, 285–296.
5 Porto, M.C.M. (1983) Physiological mechanisms of drought tolerance in cassava (Manihot esculenta Crantz). PhD dissertation. The University of Arizona, Tucson.
6 El-Sharkawy, M.A. (1993) Drought-tolerant cassava for Africa, Asia, and Latin America: breeding projects work to stabilize productivity without increasing pressures on limited natural resources. *BioScience*, **43**, 441–451.
7 Lenis, J.I., Calle, F., Jaramillo, G., Perez, J.C., Ceballo, S.H., and Cock, J.H. (2006) Leaf retention and cassava productivity. *Field Crops Res.*, **95**, 126–134.
8 El-Sharkawy, M.A. (2007) Physiological characteristics of cassava tolerance to prolonged drought in the tropics: implications for breeding cultivars adapted to seasonally dry and semiarid environments. *Braz. J. Plant Physiol.*, **19**, 257–286.
9 Fukushima, A., Kusano, M., Redestig, H., Arita, M., and Saito, K. (2009) Integrated omics approaches in plant systems biology. *Curr. Opin. Chem. Biol.*, **13** (5–6), 532–538.
10 Keurentjes, J.J.B., Koornneef, M., and Vreugdenhil, D. (2008) Quantitative genetics in the age of omics. *Curr. Opin. Plant Biol.*, **11** (2), 123–128.
11 Mochida, K., Furuta, T., Ebana, K., Shinozaki, K., and Kikuchi, J. (2009) Correlation exploration of metabolic and genomic diversity in rice. *BMC Genomics*, **10**, 568.
12 Urano, K., Kurihara, Y., Seki, M., and Shinozaki, K. (2010) "Omics" analyses of regulatory networks in plant abiotic stress responses. *Curr. Opin. Plant Biol.*, **13** (2), 132–138.
13 El-Sharkawy, M.A. (2005) How can calibrated research-based models be improved for use as a tool in identifying genes controlling crop tolerance to environmental stresses in the era of genomics – from an experimentalist's perspective. *Photosytnthetica*, **43**, 161–176.
14 IPCC – Intergovernmental Panel on Climate Change (2006) IPCC Special Report on the Regional Impacts of Climate change – An Assessment of Vulnerability. UNEP, WMO (http://www.grida.no/climate/ipcc/regional/502.htm).
15 EL-Sharkawy, M.A., De Tafur, S.M., and Cadavid, L.F. (1992) Potential photosynthesis of cassava as affected by growth conditions. *Crop Sci.*, **32** (6), 1336–1342.
16 Passioura, J.B. (1977) Grain yield, harvest index, and water use of wheat. *J. Aust. Inst. Agri. Sci.*, **43**, 117–120.
17 Sakurai, T., Plata, G., Rodríguez-Zapata, F., Seki, M., Salcedo, A., Toyoda, A., Ishiwata, A., Tohme, J., Sakaki, Y., Shinozaki, K., and Ishitani, M. (2007) Sequencing analysis of 20,000 full-length cDNA clones from cassava reveals lineage specific expansions in gene families related to stress response. *BMC Plant Biol.*, **7**, 66.
18 Bellotti, A. and Arias, B. (2001) Host plant resistance to whiteflies with emphasis on cassava as a case study. *Crop Prot.*, **20**, 813–823.
19 Burbano, M.M., Carabali, M.A., Montoya, L.J., and Belloti, A. (2007) Resistance of *Manihot* species to *Mononychellus tanajoa* (Acariformes), *Aleurotrachelus socialis*, and *Phenacoccus herreni* (Hemiptera). *Rev. Colomb. Entomol.*, **33**, 110–115.
20 Oguntunde, P.G. (2005) Whole-plant water use and canopy conductance of cassava under limited available soil water and varying evaporative demand. *Plant Soil*, **278**, 371–383.
21 El-Sharkawy, M.A. and Cock, J.H. (1987) Response of cassava to water stress. *Plant Soil*, **100**, 345–360.
22 Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. *Curr. Opin. Biotechnol.*, **17**, 113–122.
23 Xiong, L., Schumaker, K.S., and Zhu, J.-K. (2002) Cell signaling during cold, drought, and salt stress. *Plant Cell*, **14** (Suppl.), S165–S183
24 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and

tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.*, **57**, 781–803.

25 Lynch, M. and Walsh, B. (eds) (1998) *Genetics and Analysis of Quantitative Traits*, Sinuaer Associates.

26 Salvi, S. and Tuberosa, R. (2005) To clone or not to clone plant QTLs: present and future challenges. *Trends Plant Sci.*, **10**, 297–304.

27 Martienssen, R.A., Rabinowicz, P.D., O'Shaughnessy, A., and McCombie, W.R. (2004) Sequencing the maize genome. *Curr. Opin. Plant Biol.*, **7**, 102–107.

28 Van der Hoeven, R., Ronning, C., Giovannoni, J., Martin, G., and Tanksley, S. (2002) Deductions about the number, organization, and evolution of genes in the tomato genome based on analysis of a large expressed sequence tag collection and selective genomic sequencing. *Plant Cell*, **14**, 1441–1456.

29 Mochida, K. and Shinozaki, K. (2010) Genomics and bioinformatics resources for crop improvement. *Plant Cell Physiol.*, **51**, 497–523.

30 Sato, S. and Tabata, S. (2006) *Lotus japonicus* as a platform for legume research. *Curr. Opin. Plant Biol.*, **9**, 128–132.

31 Itoh, T., Tanaka, T., Barrero, R.A., Yamasaki, C., Fujii, Y., Hilton, P.B., Antonio, B.A., Aono, H., Apweiler, R., Bruskiewich, R., Bureau, T., Burr, F., Costa de Oliveira, A., Fuks, G., Habara, T., Haberer, G., Han, B., Harada, E., Hiraki, A.T., Hirochika, H., Hoen, D., Hokari, H., Hosokawa, S., Hsing, Y.-I., Ikawa, H., Ikeo, K., Imanishi, T., Ito, Y., Jaiswal, P., Kanno, M., Kawahara, Y., Kawamura, T., Kawashima, H., Khurana, J.P., Kikuchi, S., Komatsu, S., Koyanagi, K.O., Kubooka, H., Lieberherr, D., Lin, Y.-C., Lonsdale, D., Matsumoto, T., Matsuya, A., McCombie, W.R., Messing, J., Miyao, A., Mulder, N., Nagamura, Y., Nam, J., Namiki, N., Numa, H., Nurimoto, S., O'Donovan, C., Ohyanagi, H., Okido, T., Oota, S., Osato, N., Palmer, L.E., Quetier, F., Raghuvanshi, S., Saichi, N., Sakai, H., Sakai, Y., Sakata, K., Sakurai, T., Sato, F., Sato, Y., Schoof, H., Seki, M., Shibata, M., Shimizu, Y., Shinozaki, K., Shinso, Y., Singh, N.K., Smith-White, B., Takeda, J.-I., Tanino, M., Tatusova, T., Thongjuea, S., Todokoro, F., Tsugane, M., Tyagi, A.K., Vanavichit, A., Wang, A., Wing, R.A., Yamaguchi, K., Yamamoto, M., Yamamoto, N., Yu, Y., Zhang, H., Zhao, Q., Higo, K., Burr, B., Gojobori, T., and Sasaki, T. (2007) Curated genome annotation of *Oryza sativa* ssp. japonica and comparative genome analysis with *Arabidopsis thaliana*. *Genome Res.*, **17**, 175–183.

32 Neale, D.B. and Ingvarsson, P.K. (2008) Population, quantitative and comparative genomics of adaptation in forest trees. *Curr. Opin. Plant Biol.*, **11**, 149–155.

33 Zhang, P., Wang, W.-Q., Zhang, G.-L., Kaminek, M., Dobrev, P., Xu, J., and Gruissem, W. (2010) Senescence-inducible expression of isopentenyl transferase extends leaf life, increases drought stress resistance and alters cytokinin metabolism in cassava. *J. Integr. Plant Biol.*, **52**, 653–669.

34 Takei, K., Sakakibara, H., and Sugiyama, T. (2001) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. *J. Biol. Chem.*, **276**, 26405–26410.

35 Sun, J., Niu, Q.-W., Tarkowski, P., Zheng, B., Tarkowska, D., Sandberg, G., Chua, N.-H., and Zuo, J. (2003) The *Arabidopsis* AtIPT8/PGA22 gene encodes an isopentenyl transferase that is involved in *de novo* cytokinin biosynthesis. *Plant Physiol.*, **131**, 167–176.

36 Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., Tabata, S., Sandberg, G., and Kakimoto, T. (2006) Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proc. Natl. Acad. Sci. USA*, **103**, 16598–16603.

37 Sakamoto, T., Sakakibara, H., Kojima, M., Yamamoto, Y., Nagasaki, H., Inukai, Y., Sato, Y., and Matsuoka, M. (2006) Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. *Plant Physiol.*, **142**, 54–62.

38 Brugière, N., Humbert, S., Rizzo, N., Bohn, J., and Habben, J.E. (2008) A member of the maize isopentenyl transferase gene family, *Zea mays* isopentenyl transferase 2 (ZmIPT2), encodes a cytokinin biosynthetic enzyme expressed during kernel development. Cytokinin biosynthesis in maize. *Plant Mol. Biol.*, **67**, 215–229.

39 Jennings, D.L. and Iglesias, C. (2002) Breeding for crop improvement, in *Cassava: Biology, Production and Utilization* (eds R.J. Hillocks and J.M. Thresh), CABI, Oxon, pp. 149–166.

40 Kawano, K. (2003) Thirty years of cassava breeding for productivity: biological and social factors for success. *Crop Sci.*, **43**, 1325–1335.

41 Li, H.Q., Sautter, C., Potrykus, I., and Puonti-Kaerlas, J. (1996) Genetic transformation of cassava (*Manihot esculenta* Crantz). *Nat. Biotechnol.*, **14**, 736–740.

42 Taylor, N.J., Edwards, M., Kiernan, R.J., Davey, C.D., Blakesley, D., and Henshaw, G.G. (1996) Development of friable embryogenic callus and embryogenic suspension culture systems in cassava (*Manihot esculenta* Crantz). *Nat. Biotechnol.*, **14**, 726–730.

43 Vasil, I.K. (1996) Milestones in crop biotechnology: transgenic cassava and *Agrobacterium*-mediated transformation of maize. *Nat. Biotechnol.*, **14**, 702–703.

44 de Vetten, N., Wolters, A.-M., Raemakers, K., van der Meer, I., ter Stege, R., Heeres, E., Heeres, P., and Visser, R. (2003) A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nat. Biotechnol.*, **21**, 439–442.

45 Bull, S.E., Owiti, J.A., Niklaus, M., Beeching, J.R., Gruissem, W., and Vanderschuren, H. (2009) *Agrobacterium*-mediated transformation of friable embryogenic calli and regeneration of transgenic cassava. *Nat. Protoc.*, **4**, 1845–1854.

46 Andersen, M.D., Busk, P.K., Svendsen, I., and Møller, B.L. (2000) Cytochromes P-450 from cassava (*Manihot esculenta* Crantz) catalyzing the first steps in the biosynthesis of the cyanogenic glucosides linamarin and lotaustralin. Cloning, functional expression in *Pichia pastoris*, and substrate specificity of the isolated. *J. Biol. Chem.*, **275**, 1966–1975.

47 Siritunga, D. and Sayre, R. (2007) Transgenic approaches for cyanogen reduction in cassava. *J. AOAC Int.*, **90**, 1450–1455.

48 Siritunga, D. and Sayre, R. (2004) Engineering cyanogen synthesis and turnover in cassava (*Manihot esculenta*). *Plant Mol. Biol.*, **56**, 661–669.

49 Raemakers, C.J.J.M., Schreuder, M.M., Pereira, I., Suurs, L., Vincken, J.P., Jacobsen, E., and Visser, R.G.F. (2003) Production of amylose-free cassava plants by genetic modification, in *Cassava: An Ancient Crop for Modern Times Proceedings of the 5th International Meeting of the Cassava Biotechnology Network, St Louis MO, 2001* (eds C.M. Fauquet and N.J. Nigel).

50 Ihemere, U., Arias-Garzon, D., Lawrence, S., and Sayre, R. (2006) Genetic modification of cassava for enhanced starch production. *Plant Biotechnol. J.*, **4**, 453–465.

51 Ladino, J.J., Echeverry, M., Mancilla, L.I., Lopez, D., Chavarriaga, P., Tohme, J., and Roca, W. (2002) Genetic transformation of cassava: confirmation of transgenesis in clone 60444 and analysis of CRY1Ab protein in transgenic lines. Preliminary data on transformation of farmer-preferred cultivars SM1219-9 and CM3306-4. Annual Report, CIAT, Cali, Colombia.

52 Pita, J.S., Fondong, V.N., Sangaré, A., Otim-Nape, G.W., Ogwal, S., and Fauquet, C.M. (2001) Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. *J. Gen. Virol.*, **82**, 655–665.

53 Chellappan, P., Masona, M.V., Vanitharani, R., Taylor, N.J., and Fauquet, C.M. (2004) Broad spectrum resistance to ssDNA viruses associated with transgene-induced gene silencing in cassava. *Plant Mol. Biol.*, **56**, 601–611.

54 Zhang, P., Vanderschuren, H., Fütterer, J., and Gruissem, W. (2005) Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes. *Plant Biotechnol. J.*, **3**, 385–397.

55 Vanderschuren, H., Akbergenov, R., Pooggin, M.M., Hohn, T., Gruissem, W., and Zhang, P. (2007) Transgenic cassava resistance to African cassava mosaic virus is enhanced by viral DNA-A bidirectional promoter-derived siRNAs. *Plant Mol. Biol.*, **64**, 549–557.

56 Vanderschuren, H., Alder, A., Zhang, P., and Gruissem, W. (2009) Dose-dependent RNAi-mediated geminivirus resistance in the tropical root crop cassava. *Plant Mol. Biol.*, **70**, 265–272.

57 Sarria, R., Torres, E., Angel, F., Chavarriaga, P., and Roca, W.M. (2000) Transgenic plants of cassava (*Manihot esculenta*) with resistance to Basta obtained by *Agrobacterium*-mediated transformation. *Plant Cell Rep.*, **19**, 339–344.

58 Echeverry, M., Mancilla, L.I., Cortes, D.F., Chavarriaga, P., and Tohme, J. (2002) Preliminary evaluation of the expression of the transgene bar in cassava plants maintained under asexual propagation for 10 years. Annual Report, CIAT, Cali, Colombia.

59 Saelim, L., Phansiri, S., Suksangpanomrung, M., Netrphan, S., and Narangajavana, J. (2009) Evaluation of a morphological marker selection and excision system to generate marker-free transgenic cassava plants. *Plant Cell Rep.*, **28**, 445–455.

60 Cabral, G.B. and Carvalho, L.J.C.B. (2001) Analysis of proteins associated with storage root formation in cassava using two-dimensional gel electrophoresis. *Rev. Bras. Fisiol. Veg.*, **13**, 41–48.

61 Sheffield, J., Taylor, N., Fauquet, C., and Chen, S. (2006) The cassava (*Manihot esculenta* Crantz) root proteome: protein identification and differential expression. *Proteomics*, **6**, 1588–1598.

62 Mehta, A., Magalhaes, B.S., Souza, D.S.L., Vasconcelos, E.A.R., Silva, L.P., Grossi-de-Sa, M.F., Franco, O.L., da Costa, P.H.A., and Rocha, T.L. (2008) Rooteomics: the challenge of discovering plant defense-related proteins in roots. *Curr. Protein Pept. Sci.*, **9**, 108–116.

63 Baba, A., Nogueira, F., Pinheiro, C., Brasil, J., Jereissati, E., Juca, T., Soares, A., Santos, M., Domont, G., and Campos, F. (2008) Proteome analysis of secondary somatic embryogenesis in cassava (*Manihot esculenta*). *Plant Sci.*, **175**, 717–723.

64 Li, K., Zhu, W., Zeng, K., Zhang, Z., Ye, J., Ou, W., Rehman, S., Heuer, B., and Chen, S. (2010) Proteome characterization of cassava (*Manihot esculenta* Crantz) somatic embryos, plantlets and tuberous roots. *Proteome Sci.*, **8**, 10.

65 Bino, R.J., Hall, R.D., Fiehn, O., Kopka, J., Saito, K., Draper, J., Nikolau, B.J., Mendes, P., Roessner-Tunali, U., Beale, M.H., Trethewey, R.N., Lange, B.M., Wurtele, E.S., and Sumner, L.W. (2004) Potential of metabolomics as a functional genomics tool. *Trends Plant Sci.*, **9**, 418–425.

66 Roepenack-Lahaye, E., von Degenkolb, T., Zerjeski, M., Franz, M., Roth, U., Wessjohann, L., Schmidt, J., Scheel, D., and Clemens, S. (2004) Profiling of *Arabidopsis* secondary metabolites by capillary liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry. *Plant Physiol.*, **134**, 548–559.

67 Aoki-Kinoshita, K.F. and Kanehisa, M. (2007) Gene annotation and pathway mapping in KEGG. *Methods Mol. Biol.*, **396**, 71–91.

68 Kanehisa, M. (2002) The KEGG database. *Novartis Found. Symp.*, **247**, 91–101, discussion 101–103, 119–128, 244–252.

69 Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T., and Yamanishi, Y. (2008) KEGG for linking genomes to life and the environment. *Nucleic Acids Res.*, **36**, D480–D484

70 Mueller, L.A., Zhang, P., and Rhee, S.Y. (2003) AraCyc: a biochemical pathway database for *Arabidopsis*. *Plant Physiol.*, **132**, 453–460.

71 Tokimatsu, T., Sakurai, N., Suzuki, H., Ohta, H., Nishitani, K., Koyama, T., Umezawa, T., Misawa, N., Saito, K., and

Shibata, D. (2005) KaPPA-view: a web-based analysis tool for integration of transcript and metabolite data on plant metabolic pathway maps. *Plant Physiol.*, **138**, 1289–1300.

72 Sakurai, N., Ara, T., Ogata, Y., Sano, R., Ohno, T., Sugiyama, K., Hiruta, A., Yamazaki, K., Yano, K., Aoki, K., Aharoni, A., Hamada, K., Yokoyama, K., Kawamura, S., Otsuka, H., Tokimatsu, T., Kanehisa, M., Suzuki, H., Saito, K., and Shibata, D. (2010) KaPPA-View4: a metabolic pathway database for representation and analysis of correlation networks of gene co-expression and metabolite co-accumulation and omics data. *Nucleic Acids Res.*, **39**, D677–D684

73 Akiyama, K., Chikayama, E., Yuasa, H., Shimada, Y., Tohge, T., Shinozaki, K., Hirai, M.Y., Sakurai, T., Kikuchi, J., and Saito, K. (2008) PRIMe: a Web site that assembles tools for metabolomics and transcriptomics. *In Silico Biol.*, **8**, 339–345.

74 Ching, A. and Rafalski, A. (2002) Rapid genetic mapping of ESTs using SNP pyrosequencing and indel analysis. *Cell. Mol. Biol. Lett.*, **7**, 803–810.

75 Duran, C., Appleby, N., Clark, T., Wood, D., Imelfort, M., Batley, J., and Edwards, D. (2009) AutoSNPdb: an annotated single nucleotide polymorphism database for crop plants. *Nucleic Acids Res.*, **37**, D951–D953

76 Kota, R., Varshney, R.K., Prasad, M., Zhang, H., Stein, N., and Graner, A. (2008) EST-derived single nucleotide polymorphism markers for assembling genetic and physical maps of the barley genome. *Funct. Integr. Genomics*, **8**, 223–233.

77 Kota, R., Varshney, R.K., Thiel, T., Dehmer, K.J., and Graner, A. (2001) Generation and comparison of EST-derived SSRs and SNPs in barley (*Hordeum vulgare* L.). *Hereditas*, **135**, 145–151.

78 Lopez, C., Piégu, B., Cooke, R., Delseny, M., Tohme, J., and Verdier, V. (2005) Using cDNA and genomic sequences as tools to develop SNP strategies in cassava (*Manihot esculenta* Crantz). *TAG Theor. Appl. Genet.*, **110**, 425–431.

79 Rafalski, A. (2002) Applications of single nucleotide polymorphisms in crop genetics. *Curr. Opin. Plant Biol.*, **5**, 94–100.

80 Schmid, K.J., Sorensen, T.R., Stracke, R., Torjek, O., Altmann, T., Mitchell-Olds, T., and Weisshaar, B. (2003) Large-scale identification and analysis of genome-wide single-nucleotide polymorphisms for mapping in *Arabidopsis thaliana*. *Genome Res.*, **13**, 1250–1257.

81 Tang, J., Vosman, B., Voorrips, R.E., Linden, C.G., and van der Leunissen, J.A.M. (2006) QualitySNP: a pipeline for detecting single nucleotide polymorphisms and insertions/deletions in EST data from diploid and polyploid species. *BMC Bioinformatics*, **7** 438.

82 Useche, F.J., Gao, G., Harafey, M., and Rafalski, A. (2001) High-throughput identification, database storage and analysis of SNPs in EST sequences. *Genome Inform.*, **12**, 194–203.

Section IIIC Vegetable Crops: Solanaceae

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42
Tomato: Grafting to Improve Salt Tolerance

Paloma Sanchez-Bel, Isabel Egea, Francisco B. Flores, and Maria C. Bolarin

Salinity is considered one of the main factors that limit crop productivity, and development of crop species tolerant to this abiotic stress is vital to meet the growing food demand through sustainable agriculture. Therefore, the greatest challenge in the coming years is to increase crop production in such abiotic stress-affected lands as occur in areas affected by salinity. Tomato is considered one of the most economically important vegetable crops in the world, but unfortunately it is salt sensitive. The commercial success obtained through traditional breeding programs with regard to salinity tolerance has been very scarce because of the complexity of the plant response to the stress. One way of avoiding or reducing losses in production caused by salinity in high-yield genotypes would be to graft them onto rootstocks capable of reducing the effect of external salt on the shoot. This strategy could also provide the plant breeder with the possibility of combining good shoot characteristics with good root characteristics and of studying the contribution of genes transcribed in the roots to their performance on the shoot. This chapter gives an overview on the main physiological processes involved in the salt tolerance response of grafted tomato plants and illustrates how grafting can enhance salt tolerance in tomato, determined by fruit yield, a key agronomic parameter. Moreover, it is important to highlight that the salt tolerance conferred by the rootstock to the shoot genotype in terms of fruit yield seems to be a heritable trait. However, it is still necessary to conduct a good deal of research work in order to simplify the process of rootstock selection, as different results may be obtained depending on the shoot and root genotypes, as well as the salt levels and exposure times of the grafted plants.

42.1
Introduction

Tomato is considered one of the most economically important vegetable crops in the world. Abiotic stresses, like those caused by soil salinity, have a huge impact on tomato production and mainly affect arid and semiarid regions. Development of tomato plants tolerant to stress is vital to meet the growing food demand through

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sustainable agriculture. Therefore, the greatest challenge for the coming years will be to increase crop production in abiotic stress-affected lands [1].

In traditional breeding programs, commercial success has been very limited due to the complexity of the trait: salt tolerance is complex genetically and physiologically [2]. Even when halophytic species exist in the gene pool, as it is the case of tomato, the development of salt-resistant cultivars has been slow [3]. Two major problems are encountered in breeding for salt tolerance: the definition, or selection criteria, for salt tolerance and the efficient use of the wild germplasm to increase the salt tolerance of the crop [4]. The selection of appropriate genes to obtain salt-tolerant varieties is a difficult task because salt resistance is a complex character controlled by a number of genes or groups of genes and involves a number of component traits that are likely to be quantitative in nature. Thus, in the *Solanum* genus, the existence of accessions of halotolerant wild species (e.g., *Solanum pennellii*, *S. cheesmaniae*, and *S. pimpinellifolium*), and their sexual compatibility with the cultivated species (*S. lycopersicum*), should have permitted the genetic dissection of the tolerance character by traditional methods. The studies carried out to date have provided valuable information (reviewed by Cuartero *et al.* [5]). Unfortunately, despite the wealth of genetic variation within the pool of tomato wild-related species, it is still not known which are the key genes determining the high tolerance level in those plants, and it is not possible to conclude that true halotolerant cultivars have been obtained. Moreover, both the distortion of the segregation, a common fact in interspecific crosses, and the difficulties inherent to the evaluation of plants under saline conditions have made the analysis difficult. As a consequence, we still do not know which are the main genes determining salt tolerance in the wild species of tomato. The problem arising while obtaining salt-tolerant varieties is the choice of the genetic material to introduce, taking into account that new cultivars bred for salt tolerance not only have to be salt tolerant but also have to achieve the same attributes of productivity and quality as observed in modern cultivars [6, 7]. Given the rapid increase in molecular biology techniques, a key question is whether such techniques can aid the development of salt resistance in plants.

The introduction of genes conferring salt tolerance to elite cultivars or elite parents of current hybrids, by transformation, is a very attractive idea because, hypothetically, susceptible but productive cultivars should be converted into tolerant cultivars while maintaining all the very valuable traits present cultivars possess. In recent years, transgenic approaches have been employed to produce plants with enhanced salinity tolerance by overexpression of genes controlling different tolerance-related physiological mechanisms [1]. However, given the nature of the genetically complex mechanisms of abiotic stress tolerance and the potential detrimental effects, approaching this strategy with reasonably successful possibilities is extremely difficult [2, 8]. Therefore, more efforts are needed in developing transgenic tomato with overexpression/silencing of specific genes in order to evaluate their putative positive effect in enhancing drought and salt tolerance, with the final goal of circumventing the increasing problem of scarcity of water of good quality in tomato cultivation [9].

But solving a problem as complex as the profitable use of saline water in irrigated agriculture requires more than one strategy. For generating tolerant cultivars, a set of cultural techniques, each contributing to a certain degree to developing plant tolerance to the deleterious effects of salt, need to be assessed to verify the positive effects of their application [3]. Some of those strategies, such as the application of chemical fertilizers at levels somewhat above the optimum for freshwater irrigation, the application on the nutrition solution of chemical adjustment, or leaching salts to deeper soil layers have doubtful compatibility with preservation of the environment, seed priming, or seedling conditioning.

Finally, another possible cultural practice in order to avoid or reduce yield losses caused by salinity would be to graft cultivars onto rootstocks able to reduce the effect of external salt on the shoot. This strategy of grafting could also provide growers the opportunity to combine the best shoot characters with the best root characters and researchers the possibility of studying the contribution to the shoot performance of genes expressed in the roots and vice versa, and their interaction. In this respect, grafting provides an experimental means to juxtapose diverse genotypes, to test for transport of hormones, signals, or metabolites [10], and to demonstrate the mobility of RNAs and proteins through the phloem. It is important to point out that growers would exploit immediately a technique like this if that allows them to use saline conditions in the culture, while retaining the yield and quality of crop varieties for which they already have established markets.

42.2
Formation of the Rootstock–Scion Union

Grafting of two plants so that they grow as a single individual is an ancient horticultural technique that can combine valuable traits of different genotypes. Grafting is an established method of vegetative propagation for many forest trees and is widely used in horticulture to propagate ornamentals. Grafting is not limited to woody species, but it is also used for some vegetables crops [11, 12]. Among the most grafted horticultural species are the cucurbits (watermelon, melon, and cucumber) and the Solanaceae (tomato, eggplant, and pepper).

The agricultural use of grafting is considerably restricted to closely related taxa because of incompatibility. Grafting partners may belong to the same species, genus, or family. For successful grafting to take place, the vascular cambium tissues of the rootstock and scion plants must be placed physically in contact with each other. Both tissues must be kept alive until the graft has taken place, usually within a period of few weeks [13, 14].

Several authors have defined the sequence of structural events during the healing of the graft in herbaceous plants. An overview of this sequence would be as follows: (i) the scion tissue with meristematic activity is placed in intimate contact with rootstock tissue in such a manner that the cambial regions of both are able to interconnect through the callus bridge. Thus, new parenchymatous cells proliferate from both

rootstock and scion producing the callus tissue and filling up the spaces between the two components connecting the scion and the rootstock. (ii) New cambial cells differentiate from the newly formed callus, forming a continuous cambial connection between rootstock and scion. Furthermore, prior to the binding of vascular cambium across the callus bridge, initial xylem and phloem may be differentiated. The wound-repair xylem is generally the first differentiated tissue to bridge the graft union, followed by wound repair phloem. (iii) In the last step of the graft process, the newly formed cambial layer in the callus bridge begins typical cambial activity forming new vascular tissues. Production of new xylem and phloem thus permits the vascular connection between the scion and the rootstock [13, 15]. Although the time in which each process of grafting occurs depends on various factors such as the grafted species and variety, many authors observed that the differentiation of callus parenchyma to form new cambial cells begins between days 4 and 8 and is fully developed after 15 days [14, 16].

Observation of the structure of the graft union in tomato showed formation of xylem and phloem vessels through the scion–rootstock union 8 days after grafting [14]. In addition, root hydraulic conductance, L_0, indicates that the graft bond is fully functional 8 days after grafting.

Many studies have suggested that peroxidases play a role in lignification [17, 18]. Fernandez-Garcia *et al.* [14] showed that total peroxidase activity increased during development of control and grafted tomato plants. However, grafted plants showed more activity than controls, which is in accordance with the increased lignification observed in the graft union by histochemical analysis. Moreover, grafted tomato plants showed a significant increase in H_2O_2 at day 8 [14]. Lignification is a process that requires H_2O_2 and cell wall peroxidases to bring about polymerization of lignin. In addition, H_2O_2 may serve as an immediate mechanism for disease resistance in response to pathogens and it could play an important role in wound response and cell apoptosis [19–21].

Graft incompatibility includes failure to unite into a strong scion–rootstock connection, failure of the grafted plant to grow in a healthy manner, or premature death following grafting. Physiological incompatibility may be either due to lack of cellular recognition, wounding responses, and growth regulators or due to incompatible toxins, but the ultimate biological nature of this grafting incompatibility is not known [22]. In tomato, our group observed in one graft combination between two cultivars, P73 as scion and Pera as rootstock, that although grafting was successful leaf morphological alterations began to appear after a certain period of time (between 20–25 days after grafting), which seemed to be associated with hormonal imbalance (Figure 42.1). Thus, Aloni *et al.* [23] found that the disruption of rootstock–scion connections in incompatible grafting occurred approximately 25 days after grafting and proposed that the main cause of incompatibility is the occurrence of hormonal imbalance, primarily of auxins and ethylene in the root system following the establishment of grafting connections. Recently, Aloni *et al.* [13] showed a schematic presentation of a possible mechanism for grafting incompatibility in melon plants, indicating that incompatibility may result from basipetal auxin transport to the rootstock where it induces ethylene production and oxidative stress. This oxidative stress may be activated also in compatible grafting.

Figure 42.1 Leaf and apex alterations observed in tomato-grafted plants (cv. P73 used as scion and cv. Pera used as rootstock) (b–e), compared to nongrafted plants of both cultivars that showed no abnormal characteristics on such vegetative organs (a and f).

These authors suggested that exogenous application of indole acetic acid (IAA) transport blockers, abscisic acid (ABA), ethylene antagonist agents, or antioxidants may reduce grafting incompatibility by reducing oxidative stress in the root and therefore enabling its growth.

42.3
The Use of Grafting in Tomato

The application of grafting began in the 1920s, initially to limit the effects of soil pathogens such as *Fusarium oxysorum* [24]. Application and use of grafting in diverse plant species of horticultural importance have risen with the increased use of improved soil mix or substrate, farmer's preferences for better seedlings, efficient management of nursery systems, lower prices of grafted seedlings, and efficient nationwide delivery, and/or transportation system [25]. This technique has been widely used to enhance nutrient uptake [26, 27], to induce resistance against drought [28, 29], to boost resistance to low and high temperatures [29–31], to bring about resistance against heavy metal contamination [27, 32], to improve alkalinity tolerance [33], and to increase synthesis of endogenous hormones [13, 34]. Grafting has also been used as a tool to study various aspects of plant biology including apical dominance [35], nodulation [36], flowering [37], dwarfing [38], and characterization of mutants [39].

In relation to salt tolerance, it is possible to find several studies that use grafting as a tool to improve salinity tolerance of tomato either through its direct application or indirectly through the detection of QTL in support of conventional breeding [14, 40–47]. These

studies suggest that grafting provides an alternative way to improve salt tolerance by reducing the ionic stress [41, 43, 44, 48, 49] and by improving the photosynthesis performance and the antioxidant systems [47]. Cultivated tomato species is a glycophyte, thus screening for rootstocks that confer resistance to salinity to the scion is mainly directed to combinations of interspecific grafts. Tomato is compatible with a wide range of genera and species. For example, it has been grafted on potato (*S. tuberosum*) producing tomato fruits and potato tubers [50]. Although intergeneric, interspecific, and intervarietal combinations are possible, the use of halophytes as rootstocks show major problems due to the different sizes and growth rates and the compatibility between the genotypes used. Therefore, the use of interspecific hybrids as rootstocks may be a better strategy to study and integrate the agronomic, physiological, and genetic components of salt tolerance conferred by the rootstock to the scion [46]. Furthermore, grafting provides a direct way to transfer salt tolerance traits from the wild species into the domestic tomato by using recombinant inbred lines (RILs) derived from interspecific crosses as rootstocks [43]. Previous studies with tomato suggest that grafting did improve plant adaptation to salt stress [40, 41].

Grafting could be a promising tool to raise fruit quality under both nonsaline and saline conditions [32, 51, 52]. Although more studies are necessary, the results obtained by our group on this aspect are remarkable. Results obtained with tomato-grafted plants showed that grafting may be an effective agricultural approach to improve fruit quality under both control growth conditions and salinity, but careful screening for optimal rootstocks is a key question, as the fruit quality of the shoot depends, at least partially, on the root system [53]. These results are very interesting, as it is known that simultaneous increase in both fruit yield and soluble solids content, a main fruit quality parameter in tomato, in commercial tomato cultivars is a difficult task [6]. This is due to the inverse relationship generally found between both parameters, which seems to become stronger under saline conditions [54]. In this study, however, the beneficial effect induced by the rootstock on the fruit quality of the shoot genotype was not associated with any negative effect on the fruit yield under saline conditions. But the rootstock was even able to induce significant increases in fruit soluble solids content and titratable acidity not only under saline conditions but also when the grafted plants were grown under unstressful conditions [53]. These results suggest that grafting may be a valid strategy to improve fruit quality.

42.4
Physiological Processes Involved in Salt Tolerance of Grafted Tomato Plants

The salinity response of grafted plants may be different depending on the main predominant effect induced by salinity as this abiotic stress acts on plants in two ways: high concentrations of salts in soil make it harder for roots to absorb water, resulting in an osmotic stress the main symptom of which is dehydration; and high concentrations of salts within the plant can be toxic, causing an ionic stress the main symptom of which is leaf chlorosis and swelling due to the excess of sodium ion. Moreover, if the plant is not able to reach homeostasis and adapt to these salinity

conditions, oxidative stress arises as a result of the excessive production of reactive oxygen species in cells, leading to senescence and death. In a recent review of the role of grafting in plant crops under saline conditions, it was shown that grafted plants deploy numerous and diverse physiological and biochemical mechanisms in order to cope with salt stress [33].

The first phase of the plant growth response results from the effect of salt outside the plant. The immediate effect on the plant of the high concentrations of saline solutes in soil is the loss of turgor, and the main plant morphological process depending on turgor is cellular expansion. Therefore, leaf expansion and root elongation are the morphological processes that are more sensitive to the osmotic stress caused by salinity in plants. The cellular and metabolic processes involved are common to drought-affected plants. To cope with this situation, one key mechanism displayed by stressed plants with the aim of restoring the water uptake and cell turgor is osmotic adjustment. The plant needs to accumulate solutes to maintain cell volume and turgor, so the response to turgor reduction is osmotic adjustment, a major component of the response to salt stress in affected plants. The main solutes contributing to osmotic adjustment are inorganic solutes, which are taken up from the substrate and transported to the shoot, and organic solutes, which are synthesized by the plant. Na^+ and Cl^- are energetically efficient osmolytes for osmotic adjustment, but they must be compartmentalized into the vacuole to minimize ion cytotoxicity. Within the cytoplasm, osmotic adjustment is achieved by accumulation of the so-called compatible osmolytes. Some compatible osmolytes are essential elemental ions, such as K^+, but the majority of these are organic solutes, especially sugars (mainly fructose and glucose), organic acids, and other metabolites such as trehalose, proline, inositol, glycine, and betaine that have an osmoprotector role, too [55–57].

Since the long-term damage caused in cultivated tomato by salinity is ionic toxicity due to the excessive accumulation of Na^+ and Cl^- in leaves [3], it is a reasonable supposition that useful rootstocks should be able to reduce the uptake and transport rates of saline ions to the shoot (a trait often termed "salt exclusion"). The enhanced salt tolerance of grafted plants has often been associated with lower Na^+ and/or Cl^- content in the shoot [33], as has been observed in tomato [41, 43]. This indicates that tolerance induced by rootstock is related to the ionic stress rather than to the osmotic stress. However, in contrast to the cultivated species *S. lycopersicum* that generally excludes toxic ions [44, 58], most wild accessions seem to have an ionic inclusion mechanism because they accumulate higher concentrations of Na^+ and Cl^- in their leaves [59]. It is necessary to keep in mind that the use of organic solutes for osmotic adjustment is energetically much more expensive than the use of saline ions proceeding from the substrate [60]. The ATP requirement for the biosynthesis and/or transport for accumulation of solutes in leaves was assessed by Ravens [61] at 3.5 units for Na^+, 34 for mannitol, 41 for proline, 50 for glycine-betaine, and about 52 for sucrose. In this respect, salt tolerance may always be associated not only with low Na^+ concentration in the leaves but also with the capability of the tissue to tolerate Na^+. While the most tolerant genotypes of many species are those with better abilities to prevent excessive ion accumulation, the leaves of halophytes do contain

high salt concentrations [62], which are necessary to adjust the leaf water relationships with low external potentials, and halophyte plants use the cheapest solutes from an energetic point of view [63]. Tissue tolerance to Na^+ involves the storage of Na^+ in vacuoles, to avoid its accumulation in cytosol, preventing alterations in the activities of cytosolic enzymes [64]. Electrochemical H^+ gradients, generated by H^+-pumps located at the plasma membrane (H^+-ATPase) and the tonoplast (H^+-ATPase, H^+-PPase), provide the energy used by the plasma membrane- and tonoplast-bound Na^+/H^+ antiporters to couple the passive movement of H^+ to the active movement of Na^+ out of the cell and into the vacuole, respectively [65]. Such a situation seems also to occur in grafted tomato plants when grown at low-mid levels of salt, as the higher the fruit yield, the higher the contribution of inorganic solutes, including the saline ions, to the osmotic potential [43, 44]. Then, breeding for Na^+ accumulation, rather than exclusion, could be a more effective strategy for improving salt tolerance of conventional crop plants.

In addition to its known components of osmotic effect and ion toxicity, salt stress is manifested by an oxidative stress, all of which contribute to its deleterious effects [66, 67]. When plants are not able to adapt or to tolerate salinity conditions, the availability of CO_2 within the leaf is restricted and fixation of it is inhibited. This short supply of CO_2 is due to the high density of closed stomata of stressed plants. Plants tend to close stomas in response to drought and salt stress in order to avoid losing water by evaporation. The alteration in CO_2 fixation induces the impairment of ATP synthesis. Under these conditions, the concentration of the final electron acceptor $NADP^+$ is generally very low, which leads to an excess of excitation energy in the photosystems. High-energy states may be dissipated by either nonphotochemical quenching (e.g., xanthophyll cycle) or alternative processes, such as photorespiration [68]. If not dissipated, electrons accumulate in the electron transport chain and are transferred to oxygen (Mehler reaction), generating reactive oxygen species (ROS). Because of their high reactive potential, ROS are harmful to many cellular components when a certain threshold is exceeded (e.g., proteins, DNA, and lipids). This abnormal accumulation of ROS constitutes the starting point of oxidative stress. With regard to photosynthesis, under stressful conditions the electronic flow ceases and as a consequence photoinhibition is favored, but ROS accumulation also induces the photooxidation of photosystems I and II [69]. Plants have defensive mechanisms and utilize several strategies to overcome salt-mediated oxidative stress. Plant enzymatic defenses against oxidative stress include antioxidant enzymes promoting ROS scavenging such as catalases, superoxide dismutases, and peroxidases participating in the glutathione and ascorbate cycles [66, 70]. The biochemical defense system also includes nonenzymatic components such as carotenoids, ascorbate, glutathione, and tocopherols. A correlation between antioxidant capacity and salinity tolerance has been reported in tomato through comparative studies between cultivated and wild species [67, 71, 72]. According to Colla *et al.* [33], antioxidants can be used as markers of salinity tolerance in grafted vegetables. In tomato, the alleviation of oxidative damage in grafted tomato plants under NaCl stress originated from the increase in activities of catalases and enzymes involved in the ascorbate–glutathione cycle such as ascorbate peroxidase, dehydroascorbic reductase, and glutathione reductase [47].

Therefore, an efficient antioxidant system is an advantage for enhancing salt tolerance of grafted plants. Nevertheless, in contrast to the negative significance given to the increased ROS production, implying a harmful process, recent studies have shown that ROS play a key role in plants as signal transduction molecules involved in mediating adaptive responses to abiotic stress, suggesting that ROS signaling is an integral part of the response of plants to salinity [73, 74].

42.5
The Rootstock Improves Salinity Tolerance at Agronomical Level

In most studies on the role of grafting in salinity tolerance of crop plants, the salinity responses of the grafted plants have been studied mainly on the basis of plant growth but not on the basis of fruit yield [29]. Only in some cases, such as melon and cucumber, there are studies showing the salinity effect in grafted plants on fruit yield [33]. However, to our knowledge, the only results on the grafting effect in salinity tolerance of tomato on the basis of fruit yield have been obtained by our research group [40, 43, 44]. The most important question to elucidate is whether fruit yield may be increased in grafted plants grown under salinity. For example, when a commercial tomato hybrid like Jaguar was grafted onto the roots of several tomato genotypes, the positive effect of grafting on the fruit yield was found when electrical conductivity (EC) levels in the irrigation water increased (because of its increasing salt contents), with fruit yield significantly higher in all grafted combinations than that of the self-grafted cultivar (Figure 42.2). It is interesting to note that the important effect induced by some rootstocks on the salt tolerance of the shoot genotype was determined by means of fruit yield, as occurred in the plants grafted onto two of the four tomato cultivars assayed as rootstocks, Radja and Pera. Thus, while the self-grafted commercial hybrid plants reduced their fruit yield by 50% at 7.5 dS m^{-1}, the graft combinations onto Radja and Pera were able to maintain their yields around 90% at this salt level, compared to the plants grown at 2.5 dS m^{-1} (control conditions).

As we said elsewhere, although intergeneric, interspecific, and intervarietal combinations in grafting are possible, the use of halophytes as rootstocks show major problems due to the different sizes and growth rates and the compatibility between the genotypes used. Therefore, the use of interspecific hybrids as rootstocks may be a better strategy for investigating the agronomic, physiological, and genetic components of salt tolerance conferred by the rootstock to the scion. Different studies have been carried out by using a commercial tomato hybrid *S. lycopersicum* cv. Boludo as scion and as rootstock two RIL populations developed from a cross between a genotype of *S. lycopersicum* var. cerasiforme, as female parent, and as male parents two salt-tolerant lines belonging to the wild tomato species *S. pimpinellifolium* (123 lines) and *S. cheesmaniae* (100 lines). In these studies, our group corroborated the positive effect of the rootstock on fruit yield, as in both populations there were rootstock lines that raised the fruit yield of the commercial hybrid under saline conditions [46]. Taken together, the set of results obtained by our research work and discussed in this section highlights the effectiveness of grafting to enhance fruit yield in tomato.

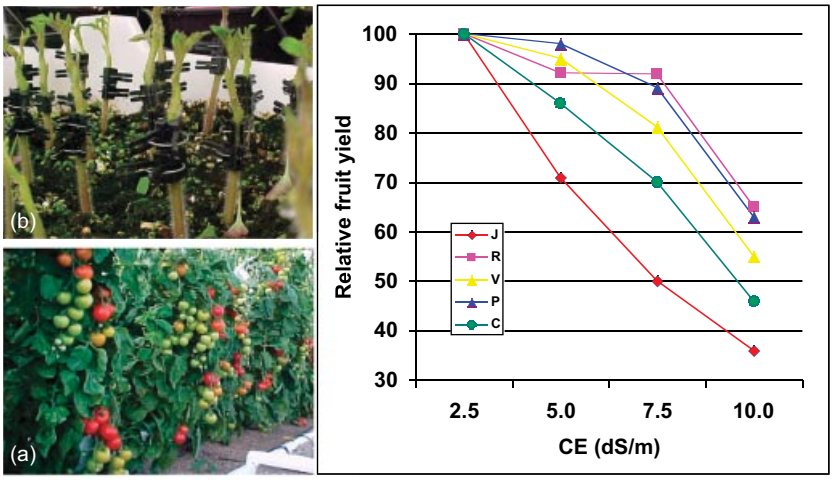

Figure 42.2 Tomato plants grafted onto different rootstocks when grafting was established (a) and at the end of growth cycle (b). Relative fruit yield comparison between self-grafted plants of a commercial tomato hybrid (cv. Jaguar, J) and Jaguar grafted onto different cultivars used as rootstocks (R, cv. Radja; V cv. Volgogradskij; P, cv. Pera; and C, commercial rootstock) at increasing levels of electrical conductivity of the irrigation solution. At the first level, the EC resulted from the sum of irrigation water EC (1.0 dS m^{-1}) plus the nutrient solution; in the following levels (5.0, 7.5, and 10.0 dS m^{-1}), the EC was increased because of the addition of 25, 50, and 75 mM NaCl, respectively, to the irrigation solution.

42.6
Genetic Basis of Salinity-Tolerant Rootstocks

The grafting strategy could also provide the plant breeder with the possibility of combining good shoot characters with good root characters and studying the contribution of genes transcribed in the roots to their performance in the shoot. An interesting approach to clarify how genes govern the tolerance to salt is the combination of segregation analysis of markers and phenotyping of lines to detect QTL. If it were possible to reveal molecular markers tightly linked to the genes governing salt tolerance, their favorable alleles could be selected in segregating populations by those markers and eventually incorporated into salt-tolerant cultivars. These markers closely linked to QTL alleles may reveal masked alleles and facilitate the introduction of genetic material without the disadvantages associated with traditional methods. The prospects of modifying a phenotype through conventional breeding have more possibilities of succeeding if it is incorporated with one or few defined regions of crucial importance than if generating a desired phenotype depends upon changes in a large number of genes, each with a small effect and scattered all over the genome. The identification of QTL has, therefore, practical importance to attempts to enhance stress tolerance.

Efforts on salt tolerance dissection using tomato experimental populations have been made taking into account different kinds of traits; however, in the case of crop plants, it is ultimately the yield under specific field conditions that will determine whether or not a gene or combination of genes (or QTL) is of agronomic importance. Experimental populations and the assays performed with them using grafting have already been discussed in the previous section, when these were used to test grafted plants with tolerance to salinity [46]. As already mentioned, salt tolerance in terms of fruit yield was studied by QTL analysis using the same RIL populations of F9 lines developed from a salt-sensitive genotype of *S. lycopersicum* var. cerasiforme, as female parent, and two salt-tolerant lines, as male parents, from *S. pimpinellifolium* and *S. cheesmaniae* [4]. Contrary to the expected, it was found that the wild allele (i.e., from the wild salt-tolerant genotype) was advantageous only at one total fruit yield QTL on chromosome 10 (*tw10.1*, near the salt-specific *fn10.1*). In fact, it was found that the advantageous allele at all fruit yield QTL came from the cultivated, salt-sensitive species. Therefore, other approaches in raising tolerance to salt using wild germplasm need to be considered.

Next, the rootstock effect on the fruit yield of a grafted tomato variety was genetically analyzed under salinity using as rootstock the previous RIL populations [46]. It is shown that the fruit yield increase induced by rootstock under salinity is a heritable trait governed by at least eight QTL. The most relevant component was the number of fruits. Thus, most of the detected QTL involved in salinity tolerance correspond to this component. In general, QTL genetic effects have a rather low degree, with contributions from 8.5 to 15.9% at most, and the advantageous allele comes from the wild, salt-tolerant species. To our knowledge, this is the first QTL analysis of the rootstock effect on the scion fruit yield. It is shown that the salt tolerance alleles from wild species can be more easily used to improve salt tolerance of the cultivated species through their utilization in tomato rootstock breeding programs [46].

42.7 Conclusions and Future Perspectives

There are rootstocks able to induce salt tolerance in tomato-grafted plants, an observation made on the basis of the determination of fruit yield, a most important agronomical trait. As discussed in this chapter, grafting practices offer the possibility of avoiding or reducing yield losses caused by salinity by means of grafting cultivars onto rootstocks able to reduce the effect of salt on the shoot. What is more, at the same time, is that it induces the development of salt tolerance to be conferred by a suitable rootstock and it allows to retain desired features of the shoot, such as fruit production and quality, which are essential parameters from the agrofood industry perspective. The main challenge here is the selection of the right rootstock, that is, the one that counteracts the negative effects of salt on the scion without affecting the levels of production and quality of the shoot. However, in order to simplify the process of rootstock selection, it would be very interesting to identify

the main rootstock characteristics able to reduce the negative effects of salinity on the shoot genotype in shorter time, in order to avoid crop losses in the grafted plants at the initial period of stress. Moreover, screening for a trait associated with a specific mechanism of the plant response to salinity is preferable to the screening for salt tolerance itself, as measuring the effect of salt on crop yield of a large number of lines is very difficult and complex. However, it is very important to take into account that the salinity response of tomato varies not only with genotype but also with salt levels and exposure times. Probably, the different and even contradictory results found in the literature may be, at least partially, due to either the stress level or the exposure time applied was not sufficient to show net differences among distinct grafted plants. Finally, the importance of osmotic tolerance mechanism to salt tolerance must be considered, which has not received as much attention as the ion exclusion mechanism, as it could be equally crucial in providing salt tolerance to tomato plants. From this perspective, more studies are required in order to arrive at the selection of the rootstock traits able to induce salt tolerance in the osmotic phase of this abiotic stress.

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References

1 Cuartero, J., Bolarin, M.C., Moreno V., and Pineda, B. (2010) Molecular tools for enhancing salinity tolerante in plants, in *Molecular Techniques for Crop Improvement.* (eds S.M. Jain and D.S. Brar), Springer Science+Business Media B.V.DOI 10.1007/978-90-481-2967-6-16.

2 Flowers, T.J. (2004) Improving crop salt tolerance. *J. Exp. Bot.*, **55**, 307–319.

3 Cuartero, J., Bolarin, M.C., Asins, M.J., and Moreno, V. (2006) Increasing salt tolerance in tomato. *J. Exp. Bot.*, **57**, 1045–1058.

4 Villalta, I., Bernet, G.P., Carbonell, E.A., and Asins, M.J. (2007) Comparative QTL analysis of salinity tolerance in terms of fruit yield using two *Solanum* populations of F7 lines. *Theor. Appl. Genet.*, **114**, 1001–1017.

5 Cuartero, J., Bolarín, M.C., Moreno, V., and Pineda, B. (2010) Molecular tools for enhancing salinity tolerance in plants, in *Molecular Techniques in Crop Improvement* (eds S.M. Jain and D.S. Brar), Springer Science Business Media BV. doi 10.1007/978-90-481-2967-6_16

6 Bai, Y. and Lindhout, P. (2007) Domestication and breeding of tomatoes: what have we gained and what can we gain in the future? *Ann. Bot.*, **100**, 1085–1094.

7 Foolad, M.R. (2007) Genome mapping and molecular breeding of tomato. *Int. J. Plant Genomics*, **2007**, Article ID 64358 52.

8 Wang, H., Miyazaki, S., Kawai, K., Deyholos, M., Galbraith, D.W., and Bohnert, H.J. (2003) Temporal progression of gene expression responses to salt shock in maize roots. *Plant Mol. Biol.*, **52**, 873–891.

9 Goel, D., Singh, A.K., Yadav, V., Babbar, S.B., and Bansal, K.C. (2010) Overexpression of osmotin gene confers

tolerance to salt and drought stresses in transgenic tomato (*Solanum lycopersicum* L.). *Protoplasma*, **245**, 133–141.
10 Turnbull, C.G.N., Booker, J.P., and Leyser, H.M.O. (2002) Micrografting techniques for testing long-distance signaling in *Arabidopsis*. *Plant J.*, **32**, 255–262.
11 Edelstein, M., Cohen, R., Shraiber, S., Pivonia, S., and Shteiberg, D. (1999) Integrated management of sudden wilt in melons, caused by *Monosporascus cannonballus*, using grafting and reduced rates of methyl bromide. *Plant Dis.*, **83**, 1142–1145.
12 Lee, J. and Oda, M. (2002) Grafting of herbaceous vegetables and ornamental crops, in *Horticultural Reviews*, vol. **28** (ed. J. Janick), Wiley-Blackwell.
13 Aloni, B., Cohen, R., Karni, L., Aktas, H., and Edelstein, M. (2010) Hormonal signaling in rootstock–scion interactions. *Sci. Hortic.*, **127**, 119–126.
14 Fernandez-Garcia, N., Martinez, V., Cerdá, A., and Carvajal, M. (2004) Fruit quality of grafted tomato plants grown under saline conditions. *J. Hort. Sci. Biotechnol.*, **79**, 995–1001.
15 Pina, A. and Errea, P. (2005) A review of new advances in mechanism of graft compatibility—incompatibility. *Sci. Hortic.*, **106**, 1–11.
16 Turquois, N. and Malone, M. (1996) Non-destructive assessment of developing hydraulic connections in the graft union of tomato. *J. Exp. Bot.*, **47**, 701–707.
17 Whetten, R.W., MacKay, J.J., and Sederoff, R.R. (1998) Recent advances in understanding lignin biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **49**, 585–609.
18 Quiroga, M., Guerrero, C., Botella, M.A., Barcelo, A., Amaya, I., Medina, M.I., Alonso, F.J., Deforchetti, S.M., Tigier, H., and Valpuesta, V. (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. *Plant Physiol.*, **122**, 1119–1127.
19 Bestwick, C.S., Brown, I.R., Bennett, M.H.R., and Manseld, J.W. (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv *phaseolicola*. *Plant Cell*, **9**, 209–221.
20 Orozco-Cardenas, M. and Ryan, C.A. (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proc. Natl. Acad. Sci. USA*, **96**, 6553–6557.
21 Pellinen, R.I., Korhonen, M.S., Tauriainen, A.A., Palva, E.T., and Kangasjarvi, J. (2002) Hydrogen peroxide activates cell death and defense gene expression in birch. *Plant Physiol.*, **130**, 549–560.
22 Flaishman, M., Loginovsky, K., Golobowich, S., and Lev-Yadun, S. (2008) *Arabidopsis thaliana* as a model system for graft union development in homografts and heterografts. *J. Plant Growth Regul.*, **27**, 231–239.
23 Aloni, B., Karni, L., Deveturero, G., Levin, Z., Cohen, R., Kazir, N., Lotan-Pompan, M., Edelstein, M., Aktas, H., Turhan, E., Joel, D.M., Horev, C., and Kapulnic, Y. (2008) Physiological and biochemical changes at the rootstock–scion interface in graft combinations between *Cucurbita* rootstocks and a melon scion. *J. Hortic. Sci. Biotechnol.*, **83**, 777–783.
24 Louws, F.J., Rivarda, C.L., and Kubota, C. (2010) Grafting fruiting vegetables to manage soil borne pathogens, foliar pathogens, arthropods and weeds. *Sci. Hortic.*, **127**, 127–146.
25 Lee, J.M., Kubota, C., Tsao, S.J., Bie, Z., Echevarria, P.H., Morra, L., and Oda, M. (2010) Current status of vegetable grafting: diffusion, grafting techniques, automation. *Sci. Hortic.*, **127**, 93–105.
26 Ahmedi, W., Nawaz, M.A., Iqbal, M.A., and Khan, M.M. (2007) Effect of different rootstocks on plant nutrient status and yield in Kinnow mandarin (*Citrus reticulata* Blanco). *Pakistan J. Bot.*, **39**, 1779–1786.
27 Savvas, D., Colla, G., Rouphael, Y., and Schwarz, D. (2010) Amelioration of heavy metal and nutrient stress in fruit vegetables by grafting. *Sci. Hortic.*, **127**, 156–161.
28 Erismann, N.D., Machado, E.C., and Tucci, M.L.S. (2008) Photosynthetic limitation by CO_2 diffusion in drought

stressed orange leaves on three rootstocks. *Photosynth. Res.*, **96**, 163–172.

29 Schwarz, D., Beuch, U., Bandte, M., Fakhro, A., Buttner, C., and Obermeier, C. (2010) Spread and interaction of Pepino mosaic virus (PepMV) and *Pythium aphanidermatum* in a closed nutrient solution recirculation system: effects on tomato growth and yield. *Plant Pathol.*, **59**, 443–452.

30 Rivero, R.M., Ruiz, J.M., Sanchez, E., and Romero, L. (2003) Does grafting provide tomato plants an advantage against H_2O_2 production under conditions of thermal shock? *Physiol. Plant.*, **117**, 44–50.

31 Venema, J.H., Dijk, B.E., Bax, J.M., van Hasselt, P.R., and Elzenga, J.T.M. (2008) Grafting tomato (*Solanum lycopersicum*) onto the rootstock of a high-altitude accession of *Solanum habrochaites* improves suboptimal-temperature tolerance. *Environ. Exp. Bot.*, **63**, 359–367.

32 Rouphael, Y., Schwarz, D., Krumbein, A., and Colla, G. (2010) Impact of grafting on product quality of fruit vegetables. *Sci. Hortic.*, **127**, 172–179.

33 Colla, G., Rouphael, Y., Leonardi, C., and Bie, Z. (2010) Role of grafting in vegetable crops grown under saline conditions. *Sci. Hortic.*, **127**, 147–155.

34 Dong, H.H., Niu, Y.H., Li, W.J., and Zhang, D.M. (2008) Effects of cotton rootstock on endogenous cytokinins and abscisic acid in xylem sap and leaves in relation to leaf senescence. *J. Exp. Bot.*, **59**, 1295–1304.

35 Mapelli, S. and Kinet, J.M. (1992) Plant growth regulator and graft control of axillary bud formation and development in the TO-2 mutant tomato. *Plant Growth Regul.*, **11**, 385–390.

36 Pedalino, M., Giller, K.E., and Kipe-Nolt, J. (1992) Genetic and physiological characterization of the non-nodulating mutant of *Phaseolus vulgaris* L. – NOD125. *J. Exp. Bot.*, **43**, 843–849.

37 Zeevaart, J.A.D. (1958) *Flower Formation as Studied by Grafting*, Mededelingen van de Landbouwhogeschool te Wageningen, The Netherlands, **58**, pp. 1–88.

38 White, R.H., Engelke, M.C., Morton, S.J., and Ruemmele, B.A. (1992) Competitive turgor maintenance in tall fescue. *Crop Sci.*, **32**, 251–256.

39 Tsukaya, N., Naito, S., Redei, G., and Komeda, Y. (1993) A new class of mutations in *Arabidopsis thaliana*, acaulis1, affecting the development of both inflorescences and leaves. *Development*, **118**, 751–764.

40 Santa-Cruz, A., Martínez-Rodríguez, M.M., Cuartero, J., and Bolarin, M.C. (2001) Response of plant yield and ion contents to salinity in grafted tomato plants. *Acta Hortic.*, **559**, 413–417.

41 Santa-Cruz, A., Martinez-Rodriguez, M.M., Perez-Alfocea, F., Romero-Aranda, R., and Bolarin, M.C. (2002) The rootstock effect on the tomato salinity response depends on the shoot genotype. *Plant Sci.*, **162**, 825–831.

42 Fernandez-Garcia, N., Martinez, V., Cerdá, A., and Carvajal, M. (2002) Water and nutrient uptake of grafted tomato plants grown under saline conditions. *J. Plant Physiol.*, **159**, 899–905.

43 Estañ, M.T., Martinez-Rodriguez, M.M., Perez-Alfocea, F., Flowers, T.J., and Bolarin, M.C. (2005) Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. *J. Exp. Bot.*, **56**, 703–712.

44 Martinez-Rodriguez, M.M., Estañ, M.T., Moyano, E., Garcia-Abellan, J.O., Flores, F.B., Campos, J.F., Al-Azzawi, M.J., Flowers, T.J., and Bolarín, M.C. (2008) The effectiveness of grafting to improve salt tolerance in tomato when an "excluder" genotype is used as scion. *Environ. Exp. Bot.*, **63**, 392–401.

45 Albacete, A., Martinez-Andujar, C., Ghanem, M.E., Acosta, M., Sanchez-Bravo, J., Asins, M.J., Cuartero, J., Lutts, S., Dodd, I.C., and Perez-Alfocea, F. (2009) Rootstock-mediated changes in xylem ionic and hormonal status are correlated with delayed leaf senescence, and increased leaf area and crop productivity in salinized tomato. *Plant Cell Environ.*, **32**, 928–938.

46 Estañ, M.T., Villalta, I., Bolarin, M.C., Carbonell, E.A., and Asins, M.J. (2009) Identification of fruit yield loci controlling the salt tolerance conferred by *Solanum*

rootstocks. *Theor. Appl. Genet.*, **118**, 305–312.

47 He, Y., Zhu, Z., Yang, J., Ni, X., and Zhu, B. (2009) Grafting increases the salt tolerance of tomato by improvement of photosynthesis and enhancement of antioxidant enzymes activity. *Environ. Exp. Bot.*, **66**, 270–278.

48 Romero, L., Belakbir, A., Ragala, L., and Ruiz, J.M. (1997) Response of plant yield and leaf pigments to saline conditions: effectiveness of different rootstocks in melon plants (*Cucumis melo* L.). *Soil Sci. Plant Nutr.*, **43**, 855–862.

49 Chen, G.X., Fu, X.P., Lips, S.H., and Sagi, M. (2003) Control of plant growth resides in the shoot, and not in the root, in reciprocal grafts of flacca and wild-type tomato (*Lysopersicon esculentum*), in the presence and absence of salinity stress. *Plant Soil*, **256**, 205–215.

50 Lee, J.M. (1994) Cultivation of grafted vegetables. I. Current status, grafting methods, and benefits. *HortScience*, **24**, 235–239.

51 Colla, G., Rouphael, Y., Cardarelli, M., Temperini, O., Rea, E., Salerno, A., and Pierandrei, F. (2008) Influence of grafting on yield and fruit quality of pepper (*Capsicum annuum* L.) grown under greenhouse conditions. *Acta Hortic.*, **782**, 359–363.

52 Proietti, S., Rouphael, Y., Colla, G., Cardarelli, M., De Agazio, M., Zacchini, M., Moscatello, S., and Battistelli, A. (2008) Fruit quality of mini-watermelon as affected by grafting and irrigation regimes. *J. Sci. Food Agric.*, **88**, 1107–1114.

53 Flores, F.B., Sanchez-Bel, P., Estañ, M.T., Martinez-Rodriguez, M.M., Moyano, E., Morales, B., Campos, J.F., Garcia-Abellán, J.O., Egea, M.I., Fernandez-Garcia, N., Romojaro, F., and Bolarin, M.C. (2010) The effectiveness of grafting to improve tomato fruit quality. *Sci. Hortic.*, **125**, 211–217.

54 Petersen, K.K., Willumsen, J., and Kaack, K. (1998) Composition and taste of tomatoes as affected by increased salinity and different salinity sources. *J. Hortic. Sci. Biotechnol.*, **73**, 205–215.

55 Ge, L., Sun, S.B., Chen, A.Q., Kapulnik, Y., and Xu, G.H. (2008) Tomato sugar transporter genes associated with mycorrhiza and phosphate. *Plant Growth Regul.*, **55**, 115–123.

56 Yang, L.F., Zhu, Y.L., Hu, C.M., Liu, Z.L., and Maruo, T. (2008) Separation and identification of specific proteins in leaves of grafted cucumber under NaCl stress. Proceedings of the International Symposium on Seed Enhancement and Seedling Production Technology, 183–190.

57 Ashraf, M. and Foolad, M.R. (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.*, **59**, 206–216.

58 Foolad, M.R. (2004) Recent advances in genetics of salt tolerance in tomato. *Plant Cell Tissue Organ Cult.*, **76**, 101–119.

59 Bolarin, M.C., Fernandez, F.G., Cruz, V., and Cuartero, J. (1991) Salinity tolerance in four wild tomato species using vegetative yield-salinity response curves. *J. Am. Soc. Hort. Sci.*, **116**, 286–290.

60 Yeo, A.R. (1983) Salinity resistance: physiologies and prices. *Physiol. Plant.*, **58**, 214–222.

61 Ravens, J.A. (1985) Regulation of pH and generation of osmolarity in vascular plants: a cost–benefit analysis in relation to efficiency of use of energy, nitrogen and water. *New Phytol.*, **101**, 25–77.

62 Santa-Cruz, A., Acosta, M., Rus, A., and Bolarin, M.C. (1999) Short-term salt tolerance mechanisms in differentially salt tolerant tomato species. *Plant Physiol. Biochem.*, **37**, 65–71.

63 Neumann, P. (1997) Salinity resistance and plant growth revisited. *Plant Cell Environ.*, **20**, 1193–1198.

64 Apse, M.P., Aharon, G.S., Snedden, W.A., and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. *Science*, **285**, 1256–1258.

65 Blumwald, E., Rea, P.A., and Poole, R.J. (1987) Preparation of tonoplast vesicles – applications to H^+-coupled secondary transport in plant vacuoles. *Methods Enzymol.*, **148**, 115–123.

66 Hernandez, S., Deleu, C., and Larher, F. (2000) Proline accumulation by leaf tissues of tomato plants in response to

salinity. *C.R. Acad Sci. III Vie*, **323**, 551–557.

67 Mittova, V., Guy, M., Tal, M., and Volokita, M. (2002) Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt-dependent oxidative stress: increased activities of antioxidant enzymes in root plastids. *Free Radic. Res.*, **36**, 195–202.

68 Saibo, N.J.M., Lourenco, T., and Oliveira, M.M. (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. *Ann. Bot.*, **103**, 609–623.

69 Asada, K. (1999) The water–water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess protons. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 601–639.

70 Hong, W.F., He, C., Wang, L., Wang, D.J., Joseph, L.M., Jantasuriyarat, C., Dai, L., and Wang, G.L. (2007) BWMK1 responds to multiple environmental stresses and plant hormones. *J. Integr. Plant Biol.*, **49**, 843–851.

71 Shalata, A., Mittova, V., Volokita, M., Guy, M., and Tal, M. (2001) Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt-dependent oxidative stress: the root antioxidative system. *Physiol. Plant.*, **112**, 487–494.

72 Zushi, K., Matsuzoe, N., and Kitano, M. (2009) Developmental and tissue-specific changes in oxidative parameters and antioxidant systems in tomato fruits grown under salt stress. *Sci. Hortic.*, **122**, 362–368.

73 Boursiac, Y., Chen, S., Luu, D.T., Sorieul, M., and van den Dries, N., and Maurel, C. (2005) Early effects of salinity on water transport in *Arabidopsis* roots. Molecular and cellular features of aquaporin expression. *Plant Physiol.*, **139**, 790–805.

74 Miller, G., Suzuki, N., Ciftci-Yilmaz, S., and Mittler, R. (2009) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.*, **33**, 453–467.

43
Tomato: Genomic Approaches for Salt and Drought Stress Tolerance

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Tomato is considered one of the most economically important vegetable crops in the world, particularly in temperate areas. Abiotic stresses as those promoted by salt accumulation and water deficiency entail significant losses of productivity. Despite the great efforts for increasing tolerance in such species of agronomic interest as tomato, the results so far obtained both with conventional breeding methods and with some biotechnological approaches have been rather scarce due to the complexity of the response to salt and drought stress. Moreover, only a small number of genes playing important roles in tolerance mechanisms to drought and/or salinity have been identified so far. Thus, novel tomato genes involved in abiotic stress tolerance need to be isolated and functionally characterized to help increase the level of salt and drought tolerance by means of gene transformation. This chapter focuses on the applications of genomic tools to the genetic dissection of those complex traits in tomato and related halotolerant wild species. First, the opportunities and limitations of the genome-wide expression profiling approaches to identify the genes associated with the stress response are discussed. Likewise, the advances achieved through forward and reverse genetics approaches such as insertional and chemical mutagenesis, TILLING, and other gene tagging approaches are reviewed. Hopefully, the combined use of all these genomics tools will lead to important advances in the genetic and physiological mechanisms of tolerance to drought and salinity in tomato, thus allowing the proper design of future breeding programs.

43.1
Introduction

Tomato is considered one of the most economically important vegetable crops in the world. Abiotic stresses, like those promoted by soil salinity and water deficiency, have a huge impact on tomato production and mainly affect arid and semiarid zones. Although production losses are very difficult to estimate, it is considered that drought

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is a major abiotic stress that affects agriculture in 45% of the world's geography, where 38% of the world population resides [1]. Moreover, 20% of the irrigated agricultural lands are considered saline, yet production losses are higher due to the continuously increasing secondary salinization brought about by the low-quality water used for irrigation [2]. This risk will increase as the population increases because cities and industry will pay for the best quality water, leaving the worst to agriculture. Development of crop plants tolerant to stress is vital to meet the growing food demand through sustainable agriculture. Therefore, the greatest challenge for the coming years will be to increase crop production in abiotic stress-affected lands.

Despite the economic relevance of tomato, the mechanisms that govern responses to these abiotic stresses in this horticultural species are not well characterized, and only a very small number of genes playing a role in tomato tolerance to salinity and drought have so far been identified. Similarly, despite the existence of a great wealth of sources of variation in salinity and drought tolerance in accessions of tomato wild species, we still do not know the main physiological processes determining their ability to grow and reproduce in stressed lands and still less about the key genes controlling the high level of tolerance. Results from several laboratories have shown that it is possible to increase the level of salt and/or drought tolerance through a transgenic approach; however, it is not possible to conclude for the moment that cultivars with a sufficient level of tolerance from an agronomic point of view have been obtained via genetic transformation. In order to overcome the present limitations, it would first be necessary to perform the genetic dissection of those complex traits in tomato and related halotolerant wild species, which in turn would enable the identification of the targets for future breeding programs. In this respect, large-scale programs based on the use of genomic approaches should usher in a new era in the knowledge of the genetic and physiological bases of the response and mechanisms of tolerance to salinity and drought, thus allowing the design of more effective strategies for breeding for abiotic stress tolerance in tomato.

43.2
Tolerance Mechanisms to Drought and Salinity in Tomato and Related Wild Species

Tolerance to drought and salt stress is a complex phenomenon at both the whole-plant level and the cellular level, and intense research efforts have focused on understanding the physiological basis of tolerance in higher plants [3–7]. In physiological terms, drought and salinity share osmotic stress, such as a decrease in soil water availability under drought or a decrease in water potential of soil solution under salinity, causing osmotic stress, which leads to decreased water uptake and loss of turgor [8]. Drought and salt stresses also provoke oxidative stress, which leads to the acceleration of reactive oxygen species (ROS) scavenging systems [9]. The differential effect induced by salinity is the toxic effect of the salt induced by the root uptake and shoot transport of saline ions. While the osmotic effect starts immediately after the imposition of salt stress (i.e., before the saline ions are taken up by the roots), the toxic effect starts later when the saline ions are transported to the shoot and build up to

toxic levels within the leaves. However, the timescale over which ion-specific damage is manifested depends on the salt sensitivity of the genotype and the stress level [10]. Although the two phases are generally separated in time for most plants, it is also possible for ion toxicity to take effect during the first phase itself and for osmotic effects to persist in the second phase [11, 12].

43.2.1
Physiological Response to Osmotic Stress Induced by Drought and Salinity

Drought and salinity are known to induce stomatal closure, slowing CO_2 assimilation and, consequently, reducing the photosynthetic rate, although the causes of decreased photosynthetic rate under abiotic stress are still not well established, and there remains substantial controversy about the main physiological targets responsible for photosynthetic impairment [13, 14]. In addition to the effects on CO_2 diffusion, ATP synthesis and reductant status, abiotic stresses can also negatively affect the Calvin cycle by reducing the content and activity of photosynthetic carbon reduction cycle enzymes, including the key enzyme Rubisco. In tomato, the photosynthetic rate and the carbohydrate availability do not seem to be the first limiting factors for plant growth under saline conditions. Rather, these are the distribution and the use of photoassimilates in the sink organs [15, 16].

One important mechanism of the maintenance of water uptake and cell turgor under drought and salinity is osmotic adjustment. One important difference between drought and salinity is the main solutes contributing to osmotic adjustment. Under drought stress, the compatible solutes (or osmolytes), together with K^+ and NO_3^-, are the most important ones contributing to the osmotic adjustment, while under salinity the most important solutes are the saline ions. The use of organic solutes for osmotic adjustment is energetically much more expensive than the use of the saline ions proceeding from the substrate [17], which could make tomato more sensitive to the osmotic component of NaCl than the ionic component [18]. Thus, in the salt-tolerant wild tomato species, the greater salt tolerance has been associated with high Na^+ accumulation in leaves and fruits [19, 20], through the use in the main of the cheapest solutes from an energetic point of view for osmotic adjustment. Within the cultivated species, the salt tolerance is not always associated with low Na^+ concentration in the leaves. Thus, a direct relationship between the fruit yield and the accumulation of leaf saline ions was found in tomato when plants are grown at low-mid levels [21]. Other evidence on the importance of the osmotic component in the salt tolerance of tomato was apparent when the salt response of tomato transgenic lines with different expression levels of *HAL1* gene, involved in Na^+ regulation, was studied. In plants of a homozygous line proceeding from a transgenic plant with a very high expression level of *HAL1* gene, the fruit yield under saline conditions not only did not increase but was also even lower than that of azygous plants, and this in spite of the much lower Na^+ uptake and Na^+ translocation to shoot maintained over time in the homozygous line [12]. The deeper physiological characterization of these plants allowed it to be elucidated that the greater ability of Na^+ exclusion in the homozygous line caused another type of osmotic problem, as leaves required an

increased synthesis of organic solutes to maintain osmotic balance, thus leading to growth penalty that negatively reflected on fruit yield. These results demonstrate the importance of considering the osmotic component of salt stress in tomato.

With respect to the role of osmolytes in drought and salinity, plants accumulate many metabolites in the cytoplasm to increase their osmotic tolerance against water loss from the cells induced by drought and salt stress, especially soluble sugars (mainly fructose and glucose) and organic acids [22]. Accumulation of compatible solutes such as proline, glycine-betaine, and trehalose has also been proposed as playing a role in tolerance to abiotic stress by protecting protein and membrane structure, regulating redox status, or acting as a scavenger of ROS [23–26]. Trehalose is present in some desiccation-tolerant higher plants [27] and the quaternary ammonium compound glycine-betaine is accumulated in numerous halophytes from several families [28–30]. Proline is one of the most studied osmolytes in tomato, as its concentration increases significantly after stress exposure, although consensus has not been reached on the relationship between stress tolerance and accumulation of proline [31]. Thus, proline increase in the leaf was deemed to be a symptom of salt injury rather than a trait of salt tolerance, whereas the opposite response was also observed, mainly when the salt tolerance mechanisms were studied at short term [32, 33]. Santa-Cruz *et al.* [34] observed a higher proline accumulation in the salt-tolerant species *Solanum pennellii* after 24 h of salt treatment, which was associated with the compensation of lower pH in the cytoplasm of the stressed cells. Taken together, the changes induced by stress in the osmolyte contents may vary depending on the intensity, duration, and progression rate of stress; proline changes may show adaptive responses of the plants in order to reestablish osmotic homeostasis at the short mid-term, but they may also show a defense strategy of the plant to tackle the harmful effects induced by stress after a long exposure and may be even part of the damage caused by the stress.

43.2.2
Physiological Response to Oxidative Stress Induced by Drought and Salinity

When plants are exposed to drought and salinity and the availability of CO_2 within the leaf is restricted and/or the synthesis of ATP is impaired, the concentration of the final electron acceptor $NADP^+$ is generally very low, which leads to an excess of excitation energy in the photosystems. High-energy states may be dissipated by either nonphotochemical quenching (e.g., xanthophyll cycle) or alternative processes, such as photorespiratory metabolism [35]. If not dissipated, electrons accumulate in the electron transport chain and are transferred to oxygen (Mehler reaction), generating ROS. Because of their high reactive potential, ROS react with, and damage, many cellular components (e.g., proteins, DNA, and lipids), constituting oxidative stress. ROS also inactivate the photochemical reaction center of PSII, causing photoinhibition. It has been proposed that most environmental stresses inactivate PSII by inhibiting the mechanisms of repairing photodamage rather than by directly attacking it [36]. Plants have defensive mechanisms and utilize several biochemical strategies to overcome drought and salt-mediated oxidative stress. Plant enzymatic defenses include antioxidant enzymes such as the phenol peroxidase, ascorbate

peroxidase, glutathione peroxidase, superoxide dismutase, and catalase that, together with other enzymes of the ascorbate-glutathione cycle, promote the scavenging of ROS [37, 38]. The biochemical defense system also includes carotenoids, ascorbate, glutathione, and tocopherols. Several authors have suggested that the function of sugars, poliols, glycine-betaine, and proline could be to protect cells against the hydroxyl radical [39]. A correlation between antioxidant capacity and salinity tolerance has been reported in tomato through comparative studies between cultivated and wild species [25, 40, 41].

In contrast to the negative term used for the increased ROS production, implying a harmful process, recent studies have shown that ROS play a key role in plants as signal transduction molecules involved in mediating responses to abiotic stress, suggesting that ROS signaling is an integral part of the adaptation response of plants to drought and salinity stresses [42, 43]. Thus, H_2O_2 seems to modulate the activities of many components that contribute to cell signaling, including Ca^{2+} and K^+ channels [44]. Although the sources of ROS under stress, mechanisms of ROS detoxification, and the role of ROS in stress signaling are all active areas of research and have been extensively studied and reviewed [43, 45], more studies are necessary before any definitive conclusion can be reached about the role of the ROS production under stress in tomato. Perhaps, ROS levels could be the key factor, producing favorable action (signaling) at low concentrations and oxidative stress at high ROS concentrations.

43.2.3
Plant Response to Ionic and Nutritional Stress Induced by Salinity

Ionic stress due to the accumulation of toxic saline ions, especially Na^+ and Cl^-, induces a nutritional stress due to the altered nutrient uptake, especially of K^+ ions. Thus, salt tolerance of the cultivated species has generally been correlated with an efficient Na^+ and Cl^- exclusion mechanism and with a better maintenance of leaf K^+ concentration. In most studies on salinity, it has not been possible to determine whether the toxic effects observed are due to Na^+ and Cl^- or both. In tomato, it is interesting to point out that similar relationship between fruit yield and leaf ionic concentrations for Na^+ and Cl^- were observed, which suggests that the toxic effects are, at least in the long term, due to the contribution of both ions [21]. Despite a wide body of literature, the mechanisms that govern tomato response to salt stress are not well characterized, and a very small number of genes playing a role in the transport of saline ions have been identified to date [46, 47]. Anion Cl^- transporters are not known in tomato yet, and the most important advances in the transport processes have been achieved for the Na^+ and K^+ transport [48, 49].

According to Plett *et al.* [50], salinity tolerance in plants is derived from the contributions of three components: tolerance to the osmotic stress imposed by salinity, exclusion of Na^+ from the shoot, and tissue tolerance of the Na^+ accumulated either by vacuolar storage or by tolerance to cytoplasmic Na^+. It is likely that all three components operate simultaneously and interact to a greater or lesser extent to provide a plant with its overall salinity tolerance. The osmotic tolerance mech-

anism for salt tolerance, which has not received as much attention as the Na^+ exclusion mechanism, appears to be equally important in providing salt tolerance to tomato plants. It has been reported that the salt-tolerant wild species and some tomato genotypes show growth stimulation on addition of NaCl to a growth medium when NaCl is rapidly accumulated and employed preferentially as an osmoticum, both in the leaves or vegetative organs [10, 21] and in the fruits [20]. Thus, the Na^+ transport to the shoot and its accumulation in leaves may be a more effective strategy than exclusion for improving tolerance of tomato when moderately saline waters are used for irrigation.

However, it is necessary to take into account that mechanisms to tolerate potentially toxic levels of Na^+ in the leaf tissues may be valid up to a certain salinity level, but not when the limit of tolerance to cytoplasmic Na^+ is exceeded. In tolerant genotypes, the Na^+ uptake was not proportional to external salinity, but was curtailed at high salinities or longer time [21, 51]. Moreover, sometimes the major differences in Na^+ accumulation are mainly observed when the concentrations are expressed on a dry weight basis instead of on a cell water basis, such as was observed by comparatively studying the response of cultivated and wild tomato species at the cell and whole-plant level [19]. Taken together, the salinity tolerance mechanisms in tomato and, especially, in wild tolerant species seem to be mainly associated with their increased capacity to uptake water throughout osmotic adjustment, diluting the toxic ions and maintaining shoot growth.

In spite of the advances in the tomato gene identification involved in the Na^+ and K^+ homeostasis in past years, more advances are necessary to understand the role and regulation of some genes involved in the reestablishment of ion homeostasis under salt stress. In this respect, tomato is a very good model for studying long distance transport of saline ions because of its physiological and anatomical structure, as tomatoes have ways of partitioning the salt arriving at the shoot, either retaining it in the leaf base or stem and preventing Na^+ from reaching the photosynthetic tissues or directing salt away from younger leaves toward older ones [10]. Recently, Olías *et al*. [49] showed that the relevant role of *SlSOS1* gene was associated with the partitioning of Na^+ in plant tomato shoot. Another important factor is to maintain a low ratio of Na^+ to K^+ reaching the shoot tissues, although tomato appears to have a poor correlation between salinity tolerance and Na^+/K^+ ratio [12, 52]. However, K^+ ions are one of the essential elements required for growth, as alterations in K^+ can disturb the osmotic balance and the function of stomata and some enzymes, and consequently more advances are necessary in order to fully understand the transport processes of this important nutrient under stress conditions [53].

43.2.4
Long-Distance Signaling Pathways and their Relationship with Drought and Salinity Response

There is evidence for a variety of long-distance signaling pathways, involving hormones and nutrient ions moving in the xylem sap, which regulate the plant growth under abiotic stress [54, 55]. It is well known that drought and salinity induce

stomatal closure and that this process is mediated by abscisic acid (ABA) and possibly by other signals generated in response to abiotic stress [35]. It has been suggested that earlier and higher ABA accumulation at short term is related to increased tolerance to drought stress. In support of results for a growth-promoting role of ABA in tomato, Makela *et al*. [56] showed that shoot growth of an ABA-deficient tomato mutant was affected by salt stress during the first phase (osmotic phase) to a significantly greater extent than its ABA-producing wild type. In a recent study, the higher drought tolerance induced by the overexpression of a tomato dehydrin (*TAS14*) was associated with a rapid increase in ABA in leaves [12], which corroborates the role of ABA in the tolerance. The action of ABA may be involved in the suppression of ethylene production [57–59]. Moreover, ABA-induced accumulation of compatible solutes, as proline, can be crucial for dehydration avoidance [60]. Thus, at the level of the organism, it seems that a main function of ABA is to coordinate the various aspects of abiotic stress response [31, 61].

43.2.5
Tolerance to Drought and Salinity Varies with the Developmental Stage

An important factor to be taken into consideration at the time of evaluating the tolerance to drought and salinity is that the tolerance at one stage of plant development is often not correlated with tolerance at other developmental stages [10]. Thus, efforts have been made to identify QTL for salinity tolerance during seed germination, vegetative growth, and later stages in tomato [46]. The overall results support the suggestion that different genetic and physiological mechanisms contribute to salt tolerance during different stages of plant development. This complicates indirect selection and comparison of results coming from different experiments and researchers. Furthermore, it requires knowledge of the physiological traits contributing to the tolerance at different plant developmental stages.

In comparison to the research conducted during seed germination and the vegetative stage, limited research has been conducted to identify QTL for salt tolerance during reproductive development in tomato [62, 63]. It is interesting to point out that a QTL involved in Na^+ accumulation has been recently identified in RIL lines proceeding from the cross between the cultivated and the wild species *S. cheesmaniae* [52]. For osmotic stress, it would be of agricultural importance to evaluate the tolerance at vegetative and reproductive developmental stages, which are key water-demanding periods of growth. Furthermore, it should be taken into account that the incidence of stress is unpredictable and plants may be exposed to drought stress at any time during their life cycle under field conditions.

In order to answer the questions "what is known so far and what remains to be known," it is essential to recognize the important work done in the past decade, as well as the important advances described here. However, more advances are necessary to understand the mechanisms underlying drought and salinity in tomato. Moreover, to obtain the knowledge required to develop genotypes with enhanced tolerance to field conditions, it is very important to combine the descriptive power of physiological analysis with the new tools of functional genomics that have emerged in

recent years, including the high-throughput methods for transcriptomic, proteomic, and ionomic analysis. Using this integrated analysis would make it possible to elucidate the dynamics of plant metabolism in the context of the plant–environment system as a whole.

43.2.6
Tomato Genes Involved in or Related to Salinity and Drought Tolerance

Although studies in model species such as *Arabidopsis* have led to important advances in the drought and salinity tolerance, the main genes involved in the tolerance process have to be identified in crops or wild species since the role could vary according to the species, as seems to occur with the *SlSOS1* gene [64]. Recently, Nagata *et al.* [65] performed a comparative molecular biological analysis of membrane transport genes in different organisms, ranging from bacteria to animals and plants. They compared the numbers of membrane transporter genes in *Arabidopsis* and rice. According to these authors, although many transporter genes are similar in these plants, *Arabidopsis* has a more diverse array of genes for multiefflux transport and for response to stress signals, while rice has more secondary transporter genes for carbohydrate and nutrient transport. After stress perception, plants must trigger signal transduction cascades, which in turn activate stress-responsive genes and ultimately lead to changes at the physiological and biochemical levels (Figure 43.1). The majority of studies have aimed to decipher the function of genes encoding downstream components (effectors), such as those coding for antiporters, heat shock proteins, superoxide dismutases (SODs), and LEA proteins, rather than upstream components (regulators), such as those coding for transcription factors and kinases.

Since the most important effect induced by salinity for a long term is the toxic effect, most approaches have been directed to studying cation transporters and their regulation, especially the Na^+ transporter genes, such as *SOS1*, *HKT*, and *NHX*. Thus, the tomato genes belonging to the SOS pathway, homologues to *SOS1*, *SOS2*, and *SOS3* genes from *Arabidopsis*, were isolated [49, 64]. SlSOS1, which encodes a putative Na^+/H^+ antiporter from tomato, highly homologous to *AtSOS1*, seems to play a relevant role in maintaining ion homeostasis in tomato, as *SlSOS1*-silenced plants were more sensitive to salt stress than wild type (WT) and showed higher Na^+ accumulation in leaves and roots and K^+ deficiency [49]. These authors concluded that besides its main action in extruding Na^+ out of the root, *SlSOS1* is critical for the partitioning of Na^+ in plant tomato shoot, retaining Na^+ in the stems, and preventing Na^+ from reaching the photosynthetic tissues. Preliminary experiments with transgenic tomato plants constitutively overexpressing *SlSOS2* and *SlSOS3* suggest a relevant role for these genes in tomato salt tolerance [64]. On the other hand, two tomato *HKT*genes, *SlHKT1.1* and *SlHKT1.2*, encoding putative Na^+ or K^+ transporters, have also recently been isolated (Belver, unpublished results). Olías *et al.* [49] proposed that the transport function of the *SOS1* and *HKT* systems in tomato may be coordinated to achieve Na^+ and K^+ homeostasis.

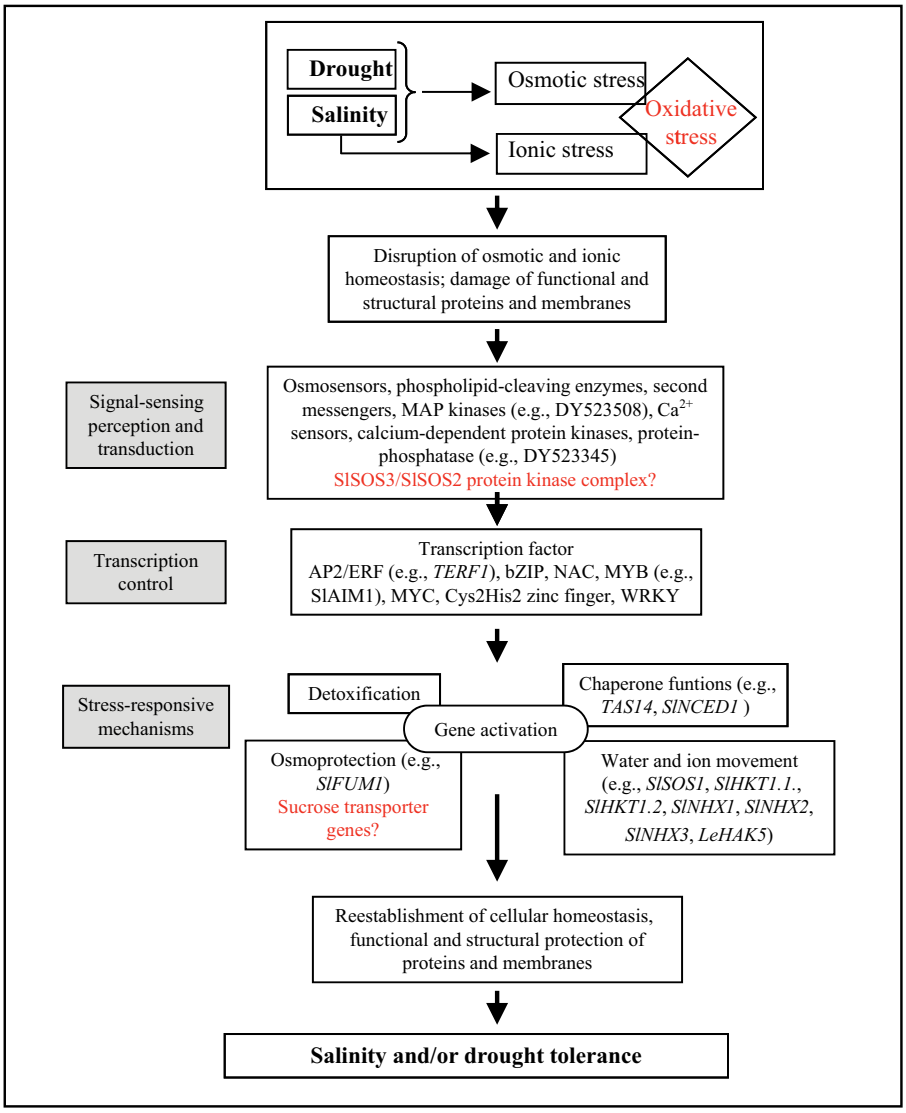

Figure 43.1 Generic pathway under salinity and drought stresses, where the regulator and response genes identified so far in tomato are included along with some genes whose role in tolerance is not yet sufficiently known.

Important work has been carried out by Pardo's Group in order to determine the role of NHX antiporters, which were thought to mediate the compartmentalization of Na^+ into vacuoles [66]. Recently, they showed that transgenic tomato plants over-expressing *AtNHX1* had larger K^+ vacuolar pools under all growth conditions tested, but no steady enhancement of Na^+ accumulation was observed under salt stress [111]. In tomato, the *SlNHX1*, *SlNHX2*, and *SlNHX3* (*LeNHX1*, *LeNHHX2*,

and *LeNHX3*, respectively, in the NCBI database) genes that encode NHX antiporters were also identified, and the function of some of them, such as SlNHX2, was studied [67].

Molecular approaches have allowed the identification of a tomato K^+ transporter, *LeHAK5* [68]. However, the low levels of the *LeHAK5* expression in K^+-starved plants grown with NaCl showed no correlation between root K^+ concentrations and transcript accumulation. The regulation of the expression of this gene was further associated with a more negative electrical potential difference across the plasma membrane of root epidermal and cortical cells, and the depolarized root plasma membrane potential of tomato plants grown with NaCl prevented the induction of *LeHAK5* produced by K^+ deprivation [48]. Therefore, under salt stress, the beneficial effect of a K^+-uptake system with a high discrimination between K^+ and Na^+ seems to be missing, although more studies are necessary in order to elucidate the main genes involved in the K^+ transport mechanisms under drought and salinity [69].

There is even more limited knowledge on the role of regulator genes, for example, protein kinases and transcription factors. In a study focused on identifying salt-responsive genes in the root tissues of tomato seedlings, 24 cDNAs corresponding to early induced transcription factors were isolated [70]. Furthermore, several components of the phosphorylation/dephosphorylation cascades, such as a protein phosphatase 2C (DY523345) and a MAPKKK (DY523508), were identified as being upregulated at the later stages of the salt stress response [70]. More recently, the ABA-induced *SlAIM1* gene, which encodes a R2R3MYB transcription factor, has also been cloned [71]. *SlAIM1* RNAi plants accumulate more Na^+, whereas the overexpression lines accumulate less Na^+ relative to wild-type plants, suggesting that *SlAIM1* regulates ion fluxes. Furthermore, a previously uncharacterized connection was observed between ABA, Na^+ homeostasis, oxidative stress, and pathogen response, suggesting *SlAIM1* has a function in both biotic and abiotic stress responses and in the existence of a crosstalk between these stress responses in tomato [71].

With respect to genes playing a role in water deficit stress tolerance of tomato, the involvement of ASR stress response proteins in physiological adaptation of wild tomato to dry climates is strongly supported by different studies [72]. Some of the most studied proteins that accumulate in response to drought stress in higher plants are the group 2 LEA proteins or dehydrins. In tomato, the expression of *TAS14* gene, which shows sequence similarities to other dehydrins, is upregulated by ABA, salt, and osmotic stress [73]. We have observed that the overexpression of *TAS14* in transgenic tomato plants improves drought and salt tolerance and that the tolerance is associated with a rapid ABA increase in the leaves of transgenic plants after applying the stress (unpublished results). In the wilted mutant, *flacca* (*flc*), induced by X-ray irradiation, it was shown that the genetic lesion impaired the last step of ABA biosynthesis. The mutant has played an invaluable role in elucidating many important features of ABA biosynthesis [74], it being known that ABA is an essential mediator in triggering the plant response to dehydration, cold, and osmotic stress. Recently, Tung *et al.* [75] cloned the tomato *SlNCED1* (*LeNCD1* in the NCBI Database) gene and demonstrated that the overexpression of this gene, which is the WT allele of

the classical ABA-deficient tomato mutant *notabilis*, enhances the water use efficiency in tomato. However, these plants exhibited important developmental alterations and greatly reduced growth, which shows the adverse consequences of a very high ABA accumulation for long term. Tung *et al*. [75] suggested that only more moderate increases in ABA biosynthesis are likely to be useful in the context of agriculture. Furthermore, valuable work has been carried out by Botella's group using mutagenesis (EMS) to identify plant genes required for salt tolerance in tomato [76, 77]. Thus, Rosado *et al*. [78] showed that crosstalk occurs between the ABA and the ethylene signaling pathways in tomato and that the *TSS2* and *TOS1* loci appear to be regulators of this crosstalk. From a spontaneous mutant (*Aco1*) of the wild tolerant species *S. pennellii*, Nunes-Nesi *et al*. [79] cloned its homologue of tomato, *SlFUM1* gene, which has an important role in the stomatal function and consequently in the osmotic tolerance.

Furthermore, several sucrose transporter genes (*LeSUT1*, *LeSUT2*, and *LeSUT4* in the NCBI database) were isolated from tomato and it has been shown that their inhibition affects tomato fruit development [80]. Although the role of sucrose transporters in the abiotic stress response of tomato has not been studied yet, these genes might be involved in the tomato responses to water stress, as plants use mainly sugars to reduce leaf osmotic potential and avoid dehydration under these conditions.

Taking into account the scant number of tomato genes identified to date in relation to salinity and, especially, water deficit stress (Figure 43.1), the identification of new genes playing pivotal roles in the response/tolerance mechanisms to drought and/or salinity is a priority objective.

43.2.7
Increasing Salt Tolerance through a Transgenic Approach: Advances and Limitations

Survey of Previous Results

In numerous papers published from the early 1990s onward, several authors have claimed enhancement of drought and salt tolerance through either overexpression of endogenous genes or, more frequently, heterologous expression of genes that supposedly act on different mechanisms involved in the process [6, 10].

Genes that have proven quite effective in providing stress tolerance using a transgenic approach belong to different categories (Table 43.1). Preliminary research in this field focused mainly on the overproduction of metabolically compatible (organic) solutes [27, 60, 81] in transgenic plants. In this respect, trehalose biosynthesis in transgenic tobacco, *Arabidopsis*, potato, or rice improved drought or salt tolerance [82]. In tomato, the yeast gene for *trehalose-6-phosphate synthase* (*TPS1*), driven by the 35S promoter of CaMV, has been used to enhance stress tolerance [83]. Under drought, salt, and oxidative stress *TPS1* tomato plants improved tolerance with respect to the wild type. However, the plants displayed abnormal phenotypes due to trehalose-6-phosphate accumulation. It has been reported that these problems can be overcome by using a microbial TPS-TPP fusion gene together with a stress-inducible promoter, directing the gene product into chloroplasts [84–87], or using a different type of trehalose biosynthetic gene (trehalose phosphorylase) that bypasses

Table 43.1 Relevant examples of genes conferring salt and drought tolerance in tomato.

Gene/Source	Function	Phenotype observed	References
TPS1/Yeast	Osmoprotection (trehalose-6 phosphate synthase)	Enhanced tolerance to drought and salt	[84]
BADH-1/Sorghum	Osmoprotection (glycine-betaine)	Maintenance of the osmotic potential under salt stress	[88]
BADH/*A. hortensis*	Osmoprotection; glycine-betaine	Improved salt tolerance	[90, 91]
KatE/*E. coli*	Oxidative stress (catalase)	Tolerance increased to the photooxidative stress induced by drought	[97]
APX/Pisum sativum	Oxidative stress (ascorbate peroxidase)	Tolerance increased to the oxidative injury induced by salt stress	[93]
HAL2/Yeast	Cation-sensitive nucleotidase required for sulfate assimilation and RNA processing	Salt tolerance in calli and rooting	[96]
HAL1/Yeast	Ion transport	Increased K^+ accumulation and higher salt tolerance under salt stress	[104, 105]
AtNHX1/*A. thaliana*	Ion transport Compartmentalization of Na^+ in vacuoles Compartmentalization of K^+ in vacuoles	High level of salt tolerance High level of salt tolerance	[109] [111]
AVP1/*A. thaliana*	Ion transport (vacuolar H^+-pyrophosphatase)	Enhanced recovery of plants in drought conditions	[117]
SlNHX2/tomato	Ion transport (tonoplast K^+/H^+ antiporter)	Silencing of *SlNHX2* increased sensitivity to NaCl	[67]
SlSOS1/tomato	Ion transport (plasma membrane Na^+/H^+ antiporter)	Silencing of *SlSOS1* increased sensitivity to NaCl	[49]
CBF1/*A. thaliana*	Transcriptional regulation (CRT/DRE-binding protein)	Enhanced tolerance to chilling, water deficit, and salt stress	[149–151]
CaKR1/pepper	Transcriptional regulation (Ankyrin repeat domain zinc finger)	Enhanced tolerance to salt and oxidative stress	[133]
Osmyb4/rice	Transcriptional regulation (MYB transcription factor)	Enhanced tolerance to drought stress and virus disease	[134]

the trehalose-6-phosphate [89]. On the other hand, glycine-betaine has been shown to protect higher plants against salt/osmotic stresses by playing an osmolyte role and protecting the photosystem II (PSII) complex under salinity [24]. In tomato, it has been reported that transgenic plants with the BADH gene from *Atriplex hortensis* improved salt tolerance [90]. Notably, it has been shown that the accumulation of glycine-betaine in genetically modified plants of tomato is more effective in the chloroplasts than in the cytosol [91], in a similar way to that previously observed in rice [92].

Another strategy to increase the level of salt tolerance has been the transfer of genes codifying different kinds of proteins functionally associated with the protection of macromolecules, such as LEA proteins, osmotin, chaperones, mRNA binding proteins [93–95], or with the protection of metabolism key enzymes [96].

Salinity and drought are well established as inducing oxidative stress. In tomato, the overexpression of the *Escherichia coli* catalase encoded by the *katE* gene increased the tolerance to the photooxidative stresses imposed by drought stress or chilling stress [97]. Interestingly, it has been reported that the coexpression of more than one gene involved in oxidative stress protection in both the chloroplasts and the cytosol gives rise to plants with increased tolerance to different types of abiotic stress [98–100].

Genetic manipulation with genes encoding membrane proteins involved in the uptake and transport of water and ions, such as water channel proteins and ion transporters, has been an alternative approach [101–103]. Thus, overexpression of yeast gene *HAL1*, a regulator of K^+ transport, in tomato resulted in increased K^+ accumulation and higher salt tolerance under salt stress [104, 105]. As ion transport across the tonoplast into vacuoles is energized by a proton moving force [106], the strategy based on the use of antiporters has generated large expectations in recent years. By overexpressing the vacuolar antiporter *AtNHX1*, a high level of salt tolerance was reported in genetically modified plants of *Arabidopsis* [107] and canola [108]. In tomato, Zhang and Blumwald [109] reported similar fruit yields in both 200 mM NaCl-treated transgenic plants overexpressing *AtNHX1* and the control plants grown under normal conditions (5 mM NaCl). However, these results have been questioned [10, 110] as has the action mechanism of *AtNHX1* gene [66]. Thus, Leidi *et al*. [111] have shown that the overexpression of *AtNHX1* in tomato can increase salt tolerance without enhancing Na^+ accumulation into vacuoles; these authors suggest that this tolerance derives from the significant role that the *AtNHX1* antiporter plays in K^+ homeostasis by capturing K^+ in the vacuoles.

New *AtNHX* genes have been cloned and characterized [112–115] and significant efforts have been carried out to identify the orthologous genes in different species, including tomato, and to perform the functional analysis, usually by overexpression and silencing in genetically modified plants. Recently, Rodriguez-Rosales *et al*. [67] studied the function of the tomato K^+/H^+ antiporter *LeNHX2* using 35S-driven gene overexpression of a histagged LeNHX2 protein in *Arabidopsis thaliana* and *LeNHX2* gene silencing in tomato. Transgenic *Arabidopsis* plants overexpressing the histagged tomato antiporter *LeNHX2* exhibited inhibited growth in the absence of K^+ in the growth medium, but were more tolerant to high concentrations of Na^+

than untransformed controls. When grown in the presence of NaCl, transgenic plants contained lower concentrations of intracellular Na^+, but more K^+, compared to untransformed controls. On the other hand, silencing of *LeNHX2* in tomato plants caused both significant inhibition of plant growth and fruit and seed production and an increased sensitivity to NaCl.

It has also been reported that the overexpression of a vacuolar H^+-pyrophosphatase (*AVP1*) from *A. thaliana* in transgenic plants of the same species increases the level of salt tolerance [116]. In tomato, the overexpression of *AVP1* resulted in greater pyrophosphate-driven cation transport into root vacuolar fractions, increased root biomass, and enhanced recovery of plants from an episode of soil water deficit stress. The more robust root systems allowed transgenic tomato plants to take up greater amounts of water during the imposed water deficit stress, resulting in a more favorable plant water status and less injury [117].

Likewise, a higher level of salt tolerance has been described through the overexpression of genes that codify plasma membrane Na^+/H^+ antiports cloned from different sources (e.g., *AtSOS1* from *A. thaliana*, [118]; *SOD2* from *Schizosaccharomyces pombe*, [98, 119]; *nhaA* from *E. coli* [120]; and *OsSOS1* from *Oryza sativa* [121]. Using posttranscriptional gene silencing, Olías *et al.* [49] evaluated the role played by *SlSOS1*, the functional homologue of *AtSOS1*, in salt tolerance of tomato. Transgenic tomato plants with reduced expression of *SlSOS1* exhibited reduced growth rate compared to WT plants under saline conditions.

Other targets in this field have been regulatory genes, such as transcription factors and those codifying signal transduction components or receptor-related proteins [122, 123]. Cloning of genes codifying transcription factors is a promising field, as they lie upstream with respect to many other genes. Recent research has led to the identification of several transcription factor families (e.g., AP2/ERF, bZIP, NAC, MYB, MYC, Cys2His2 zinc finger, and WRKY) that are important in regulating stress plant responses, including not only different kinds of abiotic stresses but also pathogen-induced defense responses, various physiological processes, hormonal signaling pathways, and several developmental processes [123–130]. For example, it has been documented that ERF proteins integrate signals from different plant hormone pathways and play roles in stress responses [131, 132]. Huang *et al.* [133] reported a novel member of ERF proteins from tomato designated tomato ethylene-responsive factor 1 (*TERF1*). Overexpression of *TERF1* in tobacco activated the expression of GCC box-containing pathogen-related genes and also gave rise to the typical ethylene triple response. Further investigation indicated that transgenic *TERF1* tobacco exhibited salt tolerance. In another work, Seong *et al.* [134] reported enhanced resistance to *Phytophthora infestans* and salt and oxidative stress tolerance in tomato plants overexpressing *CaKR1* gene, which encodes an ankyrin repeat domain zinc finger and is involved in transcriptional regulation in response to pathogens and abiotic stresses. Likewise, tomato plants overexpressing the rice *Osmyb4* gene, coding for a MYB transcription factor, acquired a higher tolerance to drought stress and viral disease [135].

In the search for different approaches, it has been suggested that genes codifying calcium sensors [136] or even DNA helicases and RNA helicases [137, 138] could be

involved in the salt tolerance process. The role of siRNAs under stress conditions is also under study [139, 140]. Finally, the knowledge of processes related to DNA/RNA metabolism and G-protein signaling pathways could be useful in elucidating the less known stress signaling networks and thereby be helpful in engineering salinity tolerance in crop plants [23].

Overall, the results obtained in this field show that the expression of different kinds of genes in transgenic plants can increase salinity and/or drought tolerance, at least to some extent (Table 43.1). Unfortunately, it is not possible to conclude for the moment that true tolerant cultivars (i.e., with a sufficient tolerance level from an agronomic point of view) have been obtained via transformation.

43.2.7.1 Aspects Related to the Evaluation of Transgenic Plants

When performing the evaluation of genetically modified plants or the functional analysis of a tolerance-related gene, it would be advisable to take into consideration some questions, such as the procedure for evaluating tolerance to salinity or drought, the plant material used for the evaluation, and the complexity of those traits.

Regarding the procedure for evaluating the tolerance to salinity, if the published results are scrutinized, some of the methods of evaluation of transgenic materials appear to be of doubtful value [110]. Responses to salinity are frequently studied with small samples, in the very short term, by using shock treatments and, moreover, data collected for very specific growth periods, in spite of the fact that salt sensitivity of tomato depends on the growth stage [10, 51]. The usefulness of *in vitro* tests, frequently used for the evaluation of salt tolerance, could also be questioned because transpiring conditions have a major influence on Na^+ transport and tolerance [141]. However, a clear relationship between tolerance to salinity *in vitro* (callus) and *in vivo* (plants grown in greenhouse) has been observed for cultivated and wild tomato species [105, 142]. *In vitro* tests can provide complementary information on the effect of some transgenes (e.g., genes involved in ionic homeostasis) and can be useful for the preselection of transgenic lines (if an *in vitro* and *in vivo* correlation has been previously shown), but they should not be used as the only criterion to determine the degree of salt tolerance.

The plant developmental stage can also be a critical issue for the evaluation of water deficit stress tolerance as seedlings, young (e.g., 2–4 leaves), and older plants will show different levels of relative tolerance. Drought stress can be imposed *in vitro* by raising the osmotic pressure (e.g., mannitol and sorbitol) or using, *in vivo* or *in vitro*, chemical agents (e.g., polyethylene glycol) limiting root water availability. In both cases, it is essential to avoid unnatural treatments leading to artifactual results. Drought tolerance is more frequently evaluated by reducing the level of water or mineral solution, in which case it is necessary to decide the level of water reduction, length treatment, and the nutrient supply during the stress period. Alternatively, the drought stress can be imposed as watering/dehydration cycles, in which case it is necessary to select the number of cycles and length of each cycle. Treatments performed in walking chambers may produce different results in the greenhouse. In any case, the relative humidity in the environment is of crucial importance as it affects stomatal closure and the water status of the plant. Moreover, as stated above, it

should be taken into account that in nature the incidence of stress is unpredictable and plants may be exposed to drought at any time during their life cycle. As a result, the evaluation for drought stress tolerance can be even more difficult than for salinity.

Another important aspect in the evaluation of saline or water deficit stress tolerance is the plant material to be used. The use of TG1 plants (primary transformants) is questionable because epigenetic effects (which are very important in some cases) may lead to erroneous conclusions. The evaluation in TG2 avoids the above problem, but it is necessary to take into account that this is a segregant progeny. In the authors' opinion, the best materials are the homozygous and azygous lines obtained in TG3. Thus, each homozygous line should be compared with two controls: the WT and the corresponding azygous line without the transgene. Positional effects can generate great differences in the expression of a given transgene in independent transgenic lines, indicating the necessity of selecting those with the best expression for the trait [143]. Dose effects of the transgene can be estimated by comparing the behavior of homozygous lines with that of hemizygous lines (i.e., those derived from the sexual crossing between the homozygous and the azygous lines). The relative tolerance of these lines can be estimated in the short and mid-term, although, finally, the long-term response (estimating yield with quantitative data) must also be reported.

Apart from all these considerations, in evaluating the tolerance of transgenic crops, it is important to perform long-term experiments, focus on growth and yield, and provide quantitative data [7, 110].

43.2.7.2 Overexpression versus Spatial and Temporary Modulation of Gene Expression

The choice of promoters can significantly affect the result of a transgenic manipulation [7]. Overexpression has so far been the most widely used strategy for increasing salinity or drought tolerance in transgenic plants. The underlying idea is that by overexpressing a certain gene or by expressing it in a constitutive way it would always have a positive effect on the phenotype. But increasing evidence supports the idea that sometimes strong and constitutive promoters (e.g., CaMV-35S) involve a high energetic cost and yield penalty in transgenic plants [12, 105, 143, 144] and, in other cases, the beneficial effects of the transgene are masked by pleiotropic effects derived from the use of strong promoters [145–147]. Evidence from research in this field supports the advantages of using inducible promoters [85, 130, 148, 149].

Thus, Kasuga *et al.* [147] overexpressed the cDNA encoding *DREB1a* under the control of a 35S promoter in transgenic *Arabidopsis* plants. As a result, transgenic plants exhibited improved tolerance to drought, salinity, and freezing stresses. However, constitutive expression of *DREB1a* resulted in severe growth retardation under normal growing conditions. In contrast, expression of *DREB1a* gene under the control of a stress-inducible promoter *rd29A* led to minimal effects on plant growth under normal growing conditions and provided even greater tolerance to abiotic stress treatments. Similar results have been observed in tomato. Hsieh *et al.* [150, 151] reported that the use of a 35S promoter to drive the expression of *Arabidopsis CBF1* in tomato improved tolerance to cold, drought, and salt loading,

at the expense of growth and yield under normal growth conditions. Lee *et al*. [152] expressed the *Arabidopsis CBF1* driven by three copies of an ABA-responsive complex (ABRC1) from the barley HAV22. Transgenic tomato plants exhibited enhanced tolerance to chilling, water deficit, and salt stresses in comparison to untransformed plants; but under normal growing conditions the ABRC1-CBF1 tomato plants also maintained normal growth and yield similar to the untransformed plants. Likewise, the constitutive expression of genes encoding compatible solutes often causes abnormalities in plants grown under normal conditions, for example, constitutive overproduction of molecules such a trehalose [145], polyamines [146], or mannitol [153]. The use of stress-inducible specific promoters may protect transgenic plants from such growth abnormalities [154].

When the scientific literature is critically reviewed, it is difficult to estimate the proportion of genes whose overexpression in tomato transgenic plants leads to undesirable pleiotropic effects and/or yield penalty, as in most cases data on salinity or drought tolerance of transgenic plants are not accompanied by a thorough phenotypical characterization, and, even less, fruit yield with and without stress conditions. Our results on the functional analysis of several genes putatively related to salinity and drought have revealed that the overexpression of some of them is linked to these kinds of collateral and undesirable effects while others are not. For example, as stated above, the expression of *HAL1* gene driven by 35s promoter enhanced the level of salt tolerance, but this positive effect was counteracted by yield penalty under control conditions [104]. However, the tomato transgenic plants overexpressing the dehydrin *TAS14* gene did not exhibit morphological or significant growth differences compared to wild-type plants when the former were grown under unstressed conditions, which indicates that in this case the yield was not penalized under normal conditions [12].

In any case, the use of inducible or specific promoters will be essential when tackling the cotransference and coexpression of several genes to avoid homology-based gene silencing [10, 144]. It is to be expected that the identification of new *cis*-regulator elements, which allow a proper expression in time and space, will be a major target in the near future [10, 101, 154, 155].

43.2.7.3 **Complexity of the Trait and Sources of Genetic Variation**

Salt and drought tolerance are complex traits [6, 7, 23, 156]. If one takes into account the diversity of mechanisms involved, the question that immediately arises is whether the introduction of a single gene can produce a sufficient level of tolerance or whether it is necessary to introduce several genes involved in different processes (e.g., osmotic adjustment, osmoprotection, ionic homeostasis, oxygen free radical scavenging, stress response, restoration of enzymatic activity, photorespiration, etc.). Of course, a particular gene (e.g., one that codes a transcription factor) can have a cascade effect, thus modifying the expression of many genes. Alternatively, the expression of a gene involved in the compartmentalization of ions in the vacuoles may alleviate toxic effects. Even so, it seems unlikely that a single gene could affect all the processes influenced by salinity. What is most likely is that the transfer and expression, in a coordinated way, of a series of genes, each of which

would affect one of the principal mechanisms of the process, would produce tolerant plants. The problem is that there is still no clear idea of which genes have to be transferred.

In this respect, rather than looking for salinity or drought tolerance-related genes in sensitive species, such as *Arabidopsis*, it would be better to focus on tolerant plants. As far as salinity is concerned, Flowers and Colmer [29] have recently reviewed the mechanisms of salt tolerance in halophytes, plants that are able to survive and reproduce in environments where the salt concentration is around 200 mM NaCl or more. The authors have proposed that research should be concentrated on a number of "model" (halotolerant) species that are representative of the various mechanisms that might be involved in tolerance. Nevertheless, as these halophytes are evolutionarily far from the main crop species, from a breeding point of view it would perhaps be better to take advantage of the existence of halotolerant accessions of wild species related to a given crop. In this respect, in the genus *Solanum* there are accessions of wild species (e.g., *S. pennellii*, *S. cheesmaniae*, and *S. pimpipinellifolium*) with a high level of tolerance to salinity and/or drought [10]. Unfortunately, despite the wealth of sources of variation, it is still not known which are the key genes determining the high tolerance level in those plants.

43.2.8
Genomic Tools for the Genetic Dissection of those Complex Traits

43.2.8.1 Transcriptomics, Proteomics, and other "Omic" Approaches

Some "omic" approaches should provide very useful information with respect to the genes actually involved in salinity and drought tolerance. Gene, metabolite, and protein discovery is being revolutionized through the combination of genome sequencing, microarray analysis, and other "omic" approaches [157]. Thus, transcriptomic analysis provides the expression profiles of hundreds or thousands of genes. At present, this kind of approach is being used to identify those genes that are up- or downregulated in response to saline or other types of abiotic stresses [10]. In this sense, several transcriptomic studies in model species such as *Arabidopsis* and rice have revealed new stress-related pathways in addition to the previously well-described stress-related genes [158]. Valuable information on the involvement of transcription factors in root apex response to salt stress has also been obtained in the model species *Medicago truncatula* [159]. To this purpose, the authors used two complementary transcriptomic approaches. Forty-six salt-regulated TF genes were identified using massive quantitative real-time PCR TF profiling, whereas Mt16K$^+$ microarray analysis revealed 824 genes (including 84 TF) showing significant changes in their expression in salt-treated root apexes.

In tomato, Wei *et al.* [160] observed changes in the accumulation of a number of different RNA from salt-treated and nontreated roots and identified 20 cDNAs that are responsive to salt treatment. The results indicated that the majority of the salt-induced changes in the root mRNA profile occurred in an ABA-independent manner. Using microarray analysis focused on early-response genes after salt stress in the cotyledons and shoot tip of tomato seedling (cv. Money Maker), Zhou *et al.* [161]

found 1757 genes regulated by salt, of which 563 were downregulated and 1194 were upregulated. Using a similar approach, Ouyang *et al.* [70] identified 201 nonredundant genes that were differentially expressed upon 30 min of severe salt stress in two cultivated tomato genotypes with different levels of salt tolerance. Interestingly, a large number of early-response genes regulated by salt stress encoded unknown proteins, indicating that there is still a great deal to discover about the mechanism of the salt tolerance in tomato. At present, we are studying the differences in the regulation of salt tolerance between cultivated tomato and its related wild salt-tolerant species *S. pennellii* using microarray analysis. It is interesting to point out that a higher number of genes with salinity (100 mM NaCl applied for 24 h and 7 days) changed their expression level in cultivated tomato, compared to the wild tolerant species, with a predominance of genes upregulated over genes downregulated. However, in wild species an induction of the expression of putative key genes occurred in response to saline stress, including several families of transcription factors, drought response genes, such as aquaporins and dehydrins, and genes involved in bioenergetics and membrane ion transport, including some ATPase subunits [162].

Transcriptomic analysis can be useful both for identifying new stress-related pathways and genes regulated by stress encoding unknown proteins (and putatively new functions) and for inferring the main mechanisms responsible for different stress tolerance between cultivated and wild species. Nevertheless, these methods usually lead to an overestimation of the number of genes supposedly involved, which makes the identification of relevant genes among an enormous number of other genes with purely secondary or irrelevant functions more difficult. Despite this, it is foreseeable that transcriptomics will become a valuable tool in the near future. However, in order to fulfill the expectations created in this field, it would be sensible to take into account the state of development at which the stress treatment is applied as well as the intensity and exposure time to stress. On the other hand, rather than apply these approaches to model (salt-sensitive) species, it would be better to apply them in both crop species and halotolerant accessions of related wild species and thus, by comparison, try to identify the genes responsible for tolerance [10, 163].

The rapid expansion of new molecular "omics" tools has opened up new perspectives in the identification of the major determinants involved in salt and drought tolerance. Thus, the proteomic analysis of plant under normal and stressed conditions (salt or drought stress) can play an important role in qualitatively and quantitatively studying the changes in protein expression patterns [164, 165]. There have been observed water deficit stress-induced changes in polypeptide accumulation in the leaves and roots of different species including tomato [166]. Likewise, salt stress resulted in the altered synthesis and accumulation of a number of prominent polypeptides in tomato roots [167]. Recently, Chen *et al.* [169] carried out a proteomic analysis to investigate the molecular differences between two tomato phenotypes differing in their salt tolerance to salinity. They identified 23 salt stress response proteins, classified into 6 functional categories, and almost all of these proteins increased their abundance in the salt-tolerant phenotype. These authors

also evaluated the effect of exogenously applied glycine-betaine and found that this compatible solute could alleviate the inhibition of tomato growth induced by salt stress by changing the expression abundance of six proteins in the salt-tolerant phenotype and two proteins in the salt-sensitive phenotype compared to salt-stressed seedlings.

Microarray analysis and proteomics of plant stress tolerance report on the regulation of many genes simultaneously through changes in transcript levels and protein levels, respectively. However, it has been reported that microarray studies provide no information about changes in the corresponding protein expression patterns [165]. Only poor or moderate correlation between changes in the levels of specific mRNAs and their corresponding proteins has been reported in studies involving yeast (*Saccharomyces cerevisiae*) [169] or *Arabidopsis* [170, 171]. A combination of microarray and proteomic analysis can indicate whether gene regulation is controlled at the level of transcription, translation, posttranslational modification, or protein accumulation. Although proteomics in higher plants is still in its infancy compared to prokaryotes, yeast, and humans [165], it is foreseeable that it may serve to shed light on some of the mechanisms of salt and drought stress tolerance.

Furthermore, by comparing the behavior of salt-sensitive and salt-tolerant genotypes (e.g., cultivated species versus accessions of the related tolerant wild species), ionomic approaches [172] can provide new insights both into the key mechanisms responsible for ion homeostasis and into the underlying cause of the different ability to use saline ions in the osmotic adjustment of halotolerant genotypes. Moreover, ionomics can be a valuable tool for thoroughly characterizing new mutants altered in the level of salt tolerance.

Large-scale programs based on the use of these omic approaches should usher in new era in the knowledge of the genetic and physiological basis of the response and mechanisms of tolerance to salinity and drought, thus allowing the design of more effective strategies for breeding cultivated species for abiotic stress tolerance. However, in order to fulfill these expectations it is necessary to focus these genomic tools to identify the main determinants of tolerance, avoiding background noise that can mask what actually is essential. Transcriptomic analysis has so far provided a general picture of genes that are down- or upregulated under abiotic stress situations as well as the number of genes belonging to different functional categories whose expression is significantly changed. Though this is a valuable information, it does not provide the basis to design a breeding program (e.g., via transgenesis), as it is very difficult to tackle the functional analysis of the hundreds (or thousands) of genes whose expression changes under abiotic stress. Thus, in the near future the most important challenge in transcriptomic analysis is to shed light on which are the key or pivotal genes responsible for salinity and drought tolerance. In the same way, the most important objective in proteomic and ionomic studies should be to clarify the main physiological processes determining the tolerance to those kinds of abiotic stress. In other words, research in this field must not be focused on increasing the complexity of traits that themselves are very complex but, on the contrary, on identifying the targets for future breeding programs.

43.2.8.2 Posttranscriptional Gene Silencing (PTGS) Large-Scale Programs

Major advances have been achieved in the study of mechanisms of PTGS and high-throughput systems are available to infer gene function [154, 173, 174]. For example, to help identify the functions of genes in rice, Miki and Shimamoto [175] developed a gateway vector, pANDA, for RNA interference of rice genes. Analysis of rice genes using this vector showed that suppression of mRNA expression was observed in more than 90% of transgenic plants examined. Hilson *et al.* [176] generated a collection of gene-specific tags (GSTs) representing at least 21 500 genes that can be used to create RNAi vectors for functional genomics studies. Preliminary analysis showed effective silencing for three genes coding for vacuolar-type H^+-ATPase subunit B3, a component of cellulose synthase and pentatricopeptide repeats [176]. According to Xiong and Zhu [177], RNAi is very efficient as an alternative to knockout mutants in components of stress signaling pathways. To the best of our knowledge, a similar strategy has not yet been applied to the tomato. However, we foresee that if systematically applied on a large-scale program for tomato and tolerant accessions of related wild species, this approach would be particularly valuable for the identification of genes related to stress response (cultivated species) or genes involved in different mechanisms responsible for salinity and drought tolerance (related wild species).

Moreover, after the identification through microarray analysis of the hundreds of genes that are differentially expressed under stress conditions, the use of a PTGS-based strategy could be particularly valuable in discriminating the key genes from those that have a merely secondary role in the tolerance process.

43.2.8.3 Spontaneous and Induced Mutants

In order to overcome the difficulties in performing the genetic dissection of these complex traits, the use of mutants as genomic tools needs to be one of the main research areas in the coming years. One of the key factors explaining our present knowledge in several areas of plant development lies in the detection and characterization of mutants altered in developmental traits, for example, those affected in tomato fruit development and maturation [178–181].

Mutagenesis in *Arabidopsis* has been employed to identify genes involved in salt tolerance [182] and thanks to the identification of *salt overly sensitive* (*sos*) mutants and the cloning and characterization of the *SOS* genes, an important and novel signaling pathway, called the SOS pathway that mediates ion homeostasis and salt tolerance, has been discovered [183–187]. Salt-tolerant mutants in *Arabidopsis* have likewise been obtained [188, 189].

By comparison, the number of mutants affected in the level of salt tolerance in species other than *Arabidopsis* that are already available to the scientific community is rather few. Occasional spontaneous mutants or, alternatively, those generated by chemical (e.g., EMS) or physical (e.g., X- and δ-rays or fast neutrons) methods could provide the basis for advancing in the knowledge of physiological processes related to salt tolerance. For example, by analyzing tomato salt-hypersensitive (*tss*) mutants, Borsani *et al.* [76] were able to identify two loci, the *TSS1* and *TSS2*. Of these, the *TSS1* locus is essential for potassium nutrition and salt tolerance, while *TSS2* plays an important role in the interactions between salt tolerance and abscisic acid signaling.

However, in the absence of obvious candidate genes, isolation of the gene altered in the mutant through a positional cloning strategy supposes huge effort. Fortunately, we can today overcome these problems by using alternative approaches.

43.2.8.4 Targeting Induced Local Lesions in Genomes Approaches

There is no generally applicable method as yet to generate a plant bearing a mutant allele of a concrete gene. Despite several technical advances, homologous recombination in plants is a technology that is still far from being considered efficient and applicable to all putative predicted genes of a given genome [190]. The completion of the genome sequence project of several cultivated plant species such as rice, cucumber, melon, and more recently tomato (http://solgenomics.net/) opens up a new era where high-throughput reverse genetic approaches are needed to meet the demand of functional genomics as well as new breeding programs.

Targeting induced local lesions in genomes (TILLING), initially developed for *Arabidopsis*, has been used successfully for high-throughput screening of libraries mutagenized with EMS or other chemical mutagens in several plant and animal species [191]. The TILLING method is based on the detection of single-nucleotide polymorphisms (SNPs) induced by the mutagen, which presumably affects the genes of interest. Induced mutations were initially identified by denaturing high-performance liquid chromatography (dHPLC), which detected conformational changes induced in heteroduplex originated by an SNP [192]. Sensitivity of the selection method was optimized later using mismatch-specific endonucleases CEL1 or ENDO1 [193, 194], which cleave at mismatches within heteroduplexes formed between mutant and wild-type DNA strands. Recently, two novel, sensitive, and very high-throughput mutation screening techniques used in human genetic diagnostic – conformation-sensitive capillary electrophoresis (CSCE) and high-resolution melt curve analysis (HRM) – have been successfully described to detect novel mutations in EMS mutagenized libraries of tomato [195]. The results obtained by Christian Bachem's group demonstrated that both new methodological approaches are fast and reliable and permit identification by TILLING of several new alleles in genes responsible for fruit quality such as phytoene synthase, which is involved in the synthesis of the characteristic red color of fruits, and sucrose synthase 2, a gene that participates in sucrose metabolism in young fruits. This group also identified 19 mutations in the coding sequence of the tomato proline dehydrogenase (ProDH) gene, with the aim of inactivating this proline degradation enzyme. Unfortunately, the effect of all these mutations on the sequence of the corresponding enzymes has not been reported; even if some of the mutations may be silent, due to synonymous substitution, others should be senseless or nonsense mutations. It is expected that inactivation of ProDH will increase proline content in all plant cells and the effect, if any, of proline accumulation in tomato resistance to abiotic stress will be determined.

43.2.8.5 Insertional Mutagenesis and Gene Traps

Insertional mutagenesis with T-DNA or transposable elements is a basic tool for the identification of genes and the analysis of their functions. With respect to insertional

mutagenesis with T-DNA, we can approach the tagging of genes by using a simple construction with a marker gene. In this way, the integration of T-DNA within the structural sequence or the controlling elements of a given gene will lead to its disruption and the consequent loss-of-function or, depending on the characteristics of the T-DNA-insert, gain-of-function, or change in its level of expression [196]. Detecting the mutant phenotype in TG1 (in the case of dominant, semidominant, or additive effects) or TG2 (recessive), as the gene is tagged by the T-DNA, means its cloning can be easier.

By comparison with classical insertional mutagenesis, the trapping systems [197] can be particularly useful for the identification of genes related to salt tolerance. The advantage of using enhancer, promoter, or gene traps resides in its self-dual nature. Like any other T-DNA, these traps act as insertion mutagens, and when T-DNA is integrated into an endogenous gene in the appropriate orientation, the reporter gene lies under the control of the regulatory elements of the tagged gene. Thus, by analyzing the reporter gene expression, one can get a precise picture on the space-temporal expression pattern of the endogenous gene tagged by the trap. In this respect, trapping strategies bring great advantages to insertional mutagenesis, allowing identification of functionally redundant genes, those expressed at multiple development states (generating confusion during phenotyping), genes whose disruption causes early lethality, and genes whose disruption causes such a soft phenotype that it may not be detected (in this case, the reporter expression provides a clue to identify the phenotype during evaluation). In addition, gene identification is independent of the expression level of the gene, thus avoiding the risk of rejecting low expressed genes even though they have major effects on the phenotype. Finally, this is the best way to identify genes that are activated or repressed in response to either an external stimulus or biotic and abiotic stress situations [196].

Using an enhancer trap (kindly provided by Dr. Thomas Jack, Department of Biological Sciences, Dartmouth College, USA) and a promoter trap (developed in the laboratory of Drs Rafael Lozano and Trinidad Angosto), a collection of 3500 T-DNA lines of tomato has been generated (unpublished results). Following a preliminary scrutiny of a sample of this collection of T-DNA lines, we have detected several salt-hypersensitive tomato mutants [198]. At first, we focused on the search of mutants altered in Na^+ absorption and transport since the plant needs to recover the ionic homeostasis in order to keep growing in a saline medium for a long term. One of the mutants most sensitive to ionic stress was detected by *in vitro* screening (Figure 43.2). We have also studied the effect of salinity in mutants altered on photosynthesis. Some of these mutants could help to clarify the putative role of chloroplasts in Na^+ scavenging from the cytoplasm [199]. Most of the hypersensitive tomato mutants are recessive, but there are some with dominant heredity. Among them, one mutant exhibits a hypersensitive reaction under salinity (i.e., lower growth, markedly wilted leaves, and few, seedless fruits). The tagged gene codes a MYB-type transcription factor. The involvement of MYB transcription factors in the response to these kinds of stresses has been previously described in model species [123, 159, 200, 201], although up to now there is only one MYB-TF identified in tomato with this possible function [71]. Moreover, we have cloned the tagged gene in another mutant, which

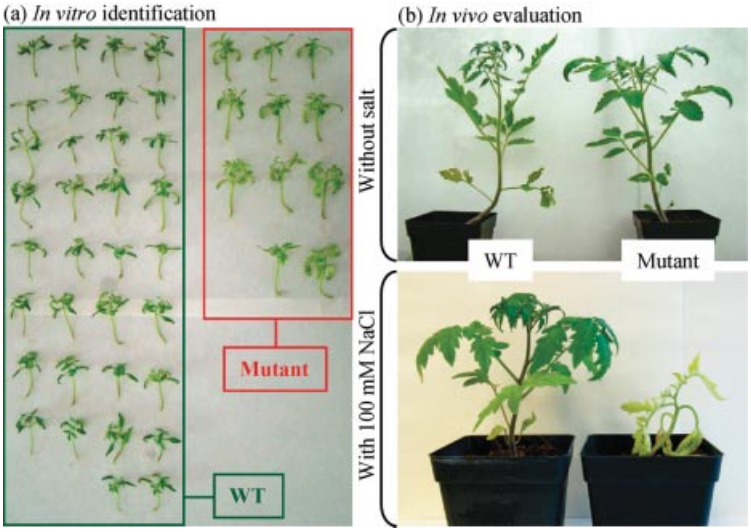

Figure 43.2 (a) A recessive mutant of tomato was identified *in vitro* (on MS medium supplemented with 100 mM of NaCl) as hypersensitive to salt. (b) The salt sensitivity of the tomato mutant was corroborated *in vivo*: Under control conditions, the phenotype and plant growth of the wild-type and tomato mutant were similar, whereas the negative effect induced by salinity was clearly greater in the mutant than in the wild type after 20 days of treatment with 100 mM of NaCl.

shows homology with a MAP-KKK protein of *Arabidopsis* that negatively regulates tolerance to salinity [202].

Apart from the insertional mutagenesis program in tomato, a cultivated species usually considered as moderately tolerant to salinity, our main objective is to identify some of the genes responsible for the high level of drought and salt tolerance in accessions of the related wild species *S. pennellii*. In order to achieve this objective, we developed an efficient transformation method (Pineda, unpublished results), which has allowed us to generate 2800 T-DNA lines of the wild species with enhancer or promoter traps. We have scrutinized T-DNA lines with reporter gene expression in stomata since the control of the stomata aperture contributes to drought tolerance [203]. We have detected a mutant of *S. pennellii* that is altered in root development and that could be interesting since tolerance to water stress is sometimes related to root mass. Moreover, we have already detected four hypersensitive *S. pennellii* mutants to water deficit stress. Interestingly, in one of these mutants the expression of the reporter gene increases after stress in stomata and transporting vessels (Figure 43.3). The scrutiny of this collection is under way to identify new mutants altered in the level of saline and water deficit stress (mainly hypersensitive). Taking into account that the collection of T-DNA lines is going to expand progressively, the identification of new drought and salt tolerance-related genes is expected in the near future.

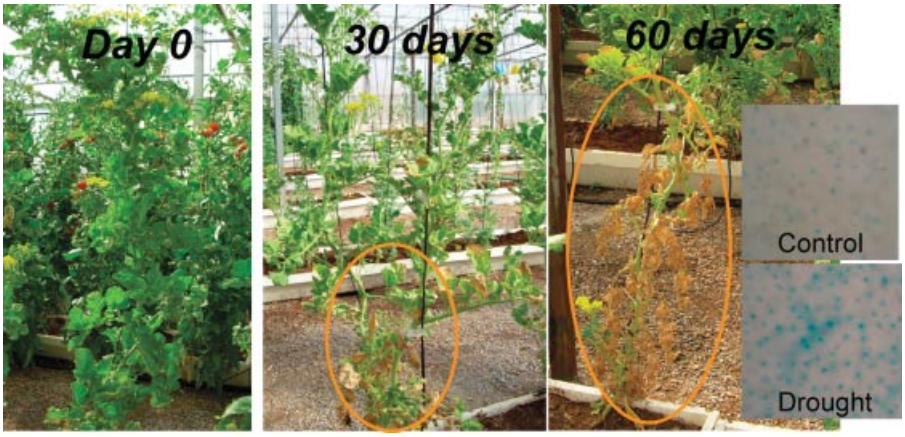

Figure 43.3 A dominant mutant of the wild species *S. pennellii* with positive GUS expression in stomata is sensitive to drought stress. The plants were subjected to several dehydration–rehydration cycles (30 and 60 days of treatment); moreover, the reporter gene (GUS) expression is enhanced in stomata after exposure to drought stress.

The identification of insertion mutants altered in the level of salt or drought tolerance may be particularly useful in the identification of key or pivotal genes involved in different tolerance mechanisms. Likewise, in-depth physiological studies, as well as the use of transcriptomic and proteomic approaches on these mutants, may provide valuable information on the key physiological processes altered in these mutants. We foresee that the combined use of all these genomics tools will allow the genetic and physiological dissection of those complex traits, thus allowing the proper design of future breeding programs.

43.3 Conclusions and Perspectives

In the genus *Solanum*, there are accessions of wild species with a high level of tolerance to salinity and drought. Unfortunately, despite the wealth of sources of variation, there is need to advance our understanding of the mechanisms underlying drought and salinity response in tomato and to elucidate how plants coordinate their responses to overcome drought and salinity. One of the main challenges for the near future is to shed light on which are the differential physiological mechanisms between cultivated and tolerant wild species under stress conditions.

Research in recent years has mainly used genetic transformation as a tool to develop stress-tolerant tomato plants. Although it has been shown that the expression of different kinds of genes in tomato transgenic plants can increase salinity and/or drought tolerance, it is not possible to conclude for the moment that true tolerant cultivars from an *agronomic* point of view have been obtained via genetic transfor-

mation. Moreover, taking into account the scant number of tomato genes identified to date in relation to salinity and, especially, water deficit stress, the identification of new genes playing a significant role in the response mechanisms to drought and/or salinity is a priority objective. To fulfill the expectations created in this field, it would be necessary to advance not only in the identification of new genes playing pivotal roles in tolerance but also in the identification of regulatory elements modulating the expression level of the transgenes spatially and temporally.

Transcriptomic analysis can be useful to identity new stress-related pathways and genes regulated by stress encoding unknown proteins (and putatively new functions) and to infer the main mechanisms responsible for stress tolerance. After the identification through microarray analysis of the hundreds of genes that are differentially expressed under stress conditions, the use of a PTGS-based strategy could be particularly valuable in order to discriminate the key genes for the tolerance processes. It is interesting to point out that a combination of microarray and proteomic analysis can indicate whether gene regulation is controlled at the level of transcription, translation, posttranslational modification, or protein accumulation. Furthermore, by comparing the behavior of salt-sensitive and salt-tolerant genotypes, ionomic approaches can provide new insight into the key mechanisms responsible for ion homeostasis and the underlying causes of the different abilities to use saline ions in the osmotic adjustment of halotolerant genotypes.

The identification of mutants, especially insertion mutants, altered in the level of salt or drought tolerance can be particularly useful in the identification of key or pivotal genes involved in different tolerance mechanisms. Likewise, in-depth physiological studies and the use of transcriptomic and proteomic approaches for these mutants may provide valuable information on the key physiological processes altered in these mutants. We foresee that the combined use of all these genomics tools will allow the genetic and physiological dissection of those complex traits, thus allowing the proper design of future breeding programs. In conclusion, the research opportunities in the coming years are very important, as not only are the -omic tools now available but also will the tomato genome be soon sequenced.

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References

1 Bot, A.J., Nachtergaele, F.O., and Young, A. (2000) Land resource potential and constraints at regional and country levels. World Soil Resources Reports 90. Land and Water Development Division, FAO, Rome.

2 Cuartero, J., Bolarin, M.C., Moreno, V. *et al.* (2008) Tolerancia a la salinidad, in *La Adptación al Ambiente y los Estreses Abióticos en la Mejora Vegetal* (eds M.T. Moreno, J.I. Cubero, S. Atienza *et al.*), Sevilla, Spain.

3 Sairam, R.K. and Tyagi, A. (2004) Physiology and molecular biology of salinity stress tolerance in plants. *Curr. Sci.*, **86**, 407–421.

4 Bartels, D. and Dunkar, R. (2005) Drought and Salt tolerance in plants. *Crit. Rev. Plant Sci.*, **24**, 23–58.

5 Foolad, M.R. (2007a) Current status of breeding tomatoes for salt and drought tolerance, in *Advances in Molecular Breeding toward Drought and Salt Tolerant Crops* (ed. M.A. Jenkes), Springer, Dordrecht, The Netherlands, pp. 669–700.

6 Cattivelli, L., Rizza, F., Badeck, F.W. *et al.* (2008) Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Res.*, **105**, 1–14.

7 Munns, R. and Tester, M. (2008) Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.*, **59**, 651–681.

8 Potters, G., Pasternak, T.P., Guisez, Y. *et al.* (2009) Different stresses, similar morphogenic responses: integrating a plethora of pathways. *Plant Cell Environ.*, **32**, 158–169.

9 Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.*, **444**, 139–158.

10 Cuartero, J., Bolarin, M.C., Asins, M.J. *et al.* (2006) Increasing salt tolerance in tomato. *J. Exp. Bot.*, **57**, 1045–1058.

11 De Costa, W., Zorb, C., Hartung, W. *et al.* (2007) Salt resistance is determined by osmotic adjustment and abscisic acid in newly developed maize hybrids in the first phase of salt stress. *Physiol. Plant.*, **131**, 311–321.

12 Muñoz-Mayor, A., Pineda, B., Garcia-Abellán, J.O. *et al.* (2008) The HAL1 function on Na^+ homeostasis is maintained over time in salt-treated transgenic tomato plants, but the high reduction of Na^+ in leaf is not associated with salt tolerance. *Physiol. Plant.*, **133**, 288–297.

13 Chaves, M.M., Flexas, J., and Pinheiro, C. (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann. Bot.*, **103**, 551–560.

14 Saibo, N.J.M., Lourencxo, T., and Oliveira, M.M. (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. *Ann. Bot.*, **103**, 609–623.

15 Balibrea, M.E., DellAmico, J., Bolarin, M.C. *et al.* (2000) Carbon partitioning and sucrose metabolism in tomato plants growing under salinity. *Physiol. Plant.*, **110**, 503–511.

16 Balibrea, M.E., Cuartero, J., Bolarin, M.C. *et al.* (2003) Sucrolytic activities during fruit development of *Lycopersicon* genotypes differing in tolerance to salinity. *Physiol. Plant.*, **118**, 38–46.

17 Neumann, P. (1997) Salinity resistance and plant growth revisited. *Plant Cell Environ.*, **20**, 1193–1198.

18 Santa-Cruz, A., Acosta, M., Perez-Alfocea, F. *et al.* (1997) Changes in free polyamine levels induced by salt stress in leaves of cultivated and wild tomato species. *Physiol. Plant.*, **101**, 341–346.

19 Rus, A., Panoff, M., Perez-Alfocea, F. *et al.* (1999) NaCl responses in tomato calli and whole plants. *J. Plant Physiol.*, **155**, 727–733.

20 Bolarin, M.C., Estañ, M.T., Caro, M. *et al.* (2001) Relation between tomato fruit growth and fruit osmotic potential under salinity. *Plant Sci.*, **160**, 1153–1159.

21 Estañ, M.T., Martinez-Rodriguez, M.M., Perez-Alfocea, F. *et al.* (2005) Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. *J. Exp. Bot.*, **56**, 703–712.

22 Cayuela, E., Perez-Alfocea, F., Caro, M. *et al.* (1996) Priming of seeds with NaCl induces physiological changes in tomato plants grown under salt stress. *Physiol. Plant.*, **96**, 231–236.

23 Tuteja, N. (2007) Mechanisms of high salinity tolerance in plants. *Meth. Enzymol.*, **428**, 419–438.

24 Türkan, I. and Demiral, T. (2009) Recent developments in understanding salinity tolerance. *Environ. Exp. Bot.*, **67**, 2–9.

25 Zushi, K. and Matsuzoe, N. (2009) Seasonal and cultivar differences in salt-induced changes in antioxidant system in tomato. *Sci. Hortic. (Amsterdam)*, **120**, 181–187.

26 Gill, S.S. and Tuteja, N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.*, **48**, 909–930.

27 Penna, S. (2003) Building stress tolerance through over-producing trehalose in transgenic plants. *Trends Plant Sci.*, **8**, 355–357.

28 Chen, T.H.H. and Murata, N. (2008) Glycinebetaine: an effective protectant against abiotic stress in plants. *Trends Plant Sci.*, **13** (9), 499–505.

29 Flowers, T.J. and Colmer, T.D. (2008) Salinity tolerance in halophytes. *New Phytol.*, **179**, 945–963.

30 Khan, M.S., Yu, X., Kikuchi, A. *et al.* (2009) Genetic engineering of glycine betaine biosynthesis to enhance abiotic stress tolerance in plants. *Plant Biotechnol.*, **26**, 125–134.

31 Ashraf, M. and Foolad, M.R. (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.*, **59**, 206–216.

32 Bolarin, M.C., Santa-Cruz, A., Cayuela, E. *et al.* (1995) Short-term solute changes in leaves and roots of cultivated and wild tomato seedlings under salinity. *J. Plant Physiol.*, **147**, 463–468.

33 Balibrea, M.E., Rus-Alvarez, A.M., Bolarin, M.C. *et al.* (1997) Fast changes in soluble carbohydrates and proline contents in tomato seedlings in response to ionic and non-ionic iso-osmotic stress. *J. Plant Physiol.*, **151**, 221–226.

34 Santa-Cruz, A., Perez-Alfocea, F., Caro, M. *et al.* (1998) Polyamines as short-term salt tolerance traits in tomato. *Plant Sci.*, **138**, 9–16.

35 Saibo, N.J.M., Lourencxo, T., and Oliveira, M.M. (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. *Ann. Bot.*, **103**, 609–623.

36 Murata, N., Takahashi, S., Nishiyama, Y. *et al.* (2007) Photoinhibition of photosystem II under environmental stress. *Biochim. Biophys. Acta*, **1767**, 414–421.

37 Hernandez, J.A., Jimenez, A., Mullineaux, P. *et al.* (2000) Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. *Plant Cell Environ.*, **23**, 853–862.

38 Hong, C., Hsu, Y.T., Tsai, Y.H. *et al.* (2007) Expression of ASCORBATE PEROXIDASE 8 in roots of rice (*Oryza sativa* L.) seedlings in response to NaCl. *J. Exp. Bot.*, **58**, 3273–3283.

39 Sickler, C.M., Edwards, G.E., Kiirats, A. *et al.* (2007) Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. *Funct. Plant Biol.* **34**, 382–391.

40 Shalata, A., Mittova, V., Volokita, M. *et al.* (2001) Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicum pennellii* to salt-dependent oxidative stress: the root antioxidative system. *Physiol. Plant.*, **112**, 487–494.

41 Mittova, V., Tal, M., Volokita, M., and Guy, M. (2002) Salt stress induces up-regulation of an efficient chloroplast antioxidant system in the salt-tolerant wild tomato species *Lycopersicon pennellii* but not in the cultivated species. *Physiol. Plant.*, **115**, 393–400.

42 Boursiac, Y., Boudet, J., Postaire, O. *et al.* (2008) Stimulus-induced downregulation of root water transport involves reactive oxygen species-activated cell signalling and plasma membrane intrinsic protein internalization. *Plant J.*, **56**, 207–218.

43 Miller, G., Suzuki, N., Ciftci-Yilmazi, S. *et al.* (2009) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.* doi: 10.1111/j.1365-3040.2009.02041.x.

44 Weinl, S. and Kudla, J. (2009) The CBL-ClPKCa^{2+}-decoding signalling network: function and perspectives. *New Phytol.*, **184**, 517–528.

45 Ben Amor, N., Jimenez, A., Megdiche, W.M. *et al.* (2007) Kinetics of antioxidant response to salinity in the halophyte *Cakile maritima*, *J. Integr. Plant Biol.*, **49**, 1–11.

46 Foolad, M.R. (2007b) Genome mapping and molecular Breeding of tomato. *Inter. J. Plant Genomics*. doi: 10.1155/2007/64358.

47 Cuartero, J., Bolarin, M.C., Moreno, V., and Pineda, B. (2010) Molecular tools for enhancing salinity tolerance in plants, in *Molecular Techniques for Crop Improvement* (eds J.S. Jain and D.S. Brar), Springer, Dordrecht, The Netherlands, pp. 373–406.

48 Nieves-Cordones, M., Miller, A.J., Aleman, F. *et al.* (2008) A putative role for the plasma membrane potential in the control of the expression of the gene encoding the tomato high-affinity potassium transporter HAK5. *Plant Mol. Biol.*, **68**, 521–532.

49 Olías, R., Eljakaoui, Z., Li, J. *et al.* (2009) Plasma membrane Na^+/H^+ antiporter SOS1 is essential in salt tolerance and affects K^+ nutrition in tomato. *Plant Cell Environ*. doi: 10.1111/j.1365-3040.2009.01971x.

50 Plett, D., Berger, B., and Tester, M. (2010) Genetic determinants of salinity tolerance in crop plants, in *Genes for Plant Abiotic Stress* (eds M.A. Jenks and A.J. Wood), Wiley Blackwell, Iowa, USA, pp. 83–111.

51 Martinez-Rodriguez, M.M., Estañ, M.T., Moyano, E. *et al.* (2008) The effectiveness of grafting to improve salt tolerance in tomato when an 'excluder' genotype is used as scion. *Environ. Exp. Bot*, **63**, 392–401.

52 Villalta, I., Reina-Sanchez, A., Cuartero, J. *et al.* (2008) Genetic analysis of Na+ and K+ concentrations in leaf and stem as physiological components of salt tolerance in Tomato. *Theor. App. Genet.*, **116**, 869–880.

53 Cuin, T.A., Betts, S.A., Chalmandrier, R. *et al.* (2008) A root's ability to retain K^+ correlates with salt tolerance in wheat. *J. Exp. Bot.*, **59**, 2697–2706.

54 Mishra, N.S., Tuteja, R., and Tuteja, N. (2006) Signaling through MAP kinase networks in plants. *Arch. Biochem. Biophys.*, **452**, 55–68.

55 Mahajan, S., Pandey, G.K., and Tuteja, N. (2008) Calcium- and salt-stress signaling in plants: shedding light on SOS pathway. *Arch. Biochem. Biophys.*, **471**, 146–158.

56 Makela, P., Munns, R., Colmer, T.D. *et al.* (2003) Growth of tomato and an ABA-deficient mutant (sitiens) under saline conditions. *Physiol. Plant.*, **117** (1), 58–63.

57 Sharp, R.E., LeNoble, M.E., Else, M.A. *et al.* (2000) Endogenous ABA maintains shoot growth in tomato independently of effects on plant water balance: evidence for an interaction with ethylene. *J. Exp. Bot.*, **51**, 1575–1584.

58 LeNoble, M.E., Spollen, W.G., and Sharp, R.E. (2004) Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression. *J. Exp. Bot.*, **55**, 237–245.

59 Thompson, A.J., Andrews, J., Mulholland, B.J. *et al.* (2007) Overproduction of abscisic acid in tomato increases transpiration efficiency and root hydraulic conductivity and influences leaf expansion. *Plant Physiol.*, **143**, 1905–1917.

60 Kavi Kishor, P.B., Sangam, S., Amrutha, R.N. *et al.* (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Curr. Sci.*, **88**, 424–438.

61 Davies, W.J., Kudoyarova, G., and Hartung, W. (2005) Long-distance ABA signaling and its relation to other signalling pathways in the detection of soil drying and the mediation of the plant's response to drought. *J. Plant Growth Reg.*, **24**, 285–295.

62 Monforte, A.J., Asíns, M.J., and Carbonell, E.A. (1996) Salt tolerance in *Lycopersicon* species. IV. Efficiency of marker-assisted selection for salt tolerance improvement. *Theor. Appl. Genet.*, **93** (5–6), 765–772.

63 Estañ, M.T., Villalta, I., Bolarin, M.C. *et al.* (2009) Identification of fruit yield loci controlling the salt tolerance conferred by

Solanum rootstocks. *Theor. App. Genet.*, **118**, 305–312.

64 Olías, R., Eljakaoui, Z., Alvarez-de-Morales, P. *et al.* (2008) Characterization of a SOS3-like calcium binding protein and SOS2-like protein kinase genes from tomato (*Solanum lycopersicum*). XVI Congress of the Federation of European Societies of Plant Biology (FESPB 2008) Tampere, Finland P09-021.

65 Nagata, T., Iizumi, S., Satoh, K. *et al.* (2008) Comparative molecular biological analysis of membrane transport genes in organisms, *Plant Mol. Biol.*, **66**, 565–585.

66 Pardo, J.M., Cubero, B., Leidi, E.O. *et al.* (2006) Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. *J. Exp. Bot.*, **57**, 1181–1199.

67 Rodríguez-Rosales, M.P., Jiang, X.J., Gálvez, F.J. *et al.* (2008) Overexpression of the tomato K^+/H^+ antiporter LeNHX2 confers salt tolerance by improving potassium compartmentalization. *New Phytol.*, **179**, 366–377.

68 Nieves-Cordones, M., Martinez-Cordero, M.A., Martinez, V. *et al.* (2007) An NH_4^+-sensitive component dominates high-affinity K^+ uptake in tomato plants. *Plant Sci.*, **172**, 273–280.

69 Szczerba, M.W., Britto, D.T., and Kronzucker, H.J. (2009) K^+ transport in plants: physiology and molecular biology. *J. Plant Physiol.*, **166**, 447–466.

70 Ouyang, B., Yang, T., Li, H. *et al.* (2007) Identification of early salt stress response genes in tomato root by suppression subtractive hybridization and microarray analysis. *J. Exp. Bot.*, **58**, 507–520.

71 AbuQamar, S., Luo, H., Laluk, K. *et al.* (2009) Crosstalk between biotic and abiotic stress responses in tomato is mediated by the AIM1 transcription factor. *Plant J.*, **58**, 347–360.

72 Bermudez-Morettia, M., Maskinb, L., Gudesblatb, G. *et al.* (2006) ASR1, a stress-induced tomato protein, protects yeast from osmotic stress. *Physiol. Plant.*, **127**, 111–118.

73 Godoy, J.A., Lunar, R., Torresschumann, S. *et al.* (1994) Expression, tissue distribution and subcellular-localization of dehydrin Tas14 in salt-stressed tomato plants. *Plant Mol. Biol.*, **26**, 1921–1934.

74 Taylor, I.B., Sonneveld, T., Bugg, T.D.H. *et al.* (2005) Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. *J. Plant Growth Regul.*, **24**, 253–273.

75 Tung, S., Smeeton, R., White, C.A. *et al.* (2008) Over-expression of LeNCED1 in tomato (*Solanum lycopersicum* L.) with the rbcS3C promoter allows recovery of lines that accumulate very high levels of abscisic acid and exhibit severe phenotypes. *Plant Cell Environ.*, **31**, 968–981.

76 Borsani, O., Cuartero, J., Fernandez, J.A. *et al.* (2001) Identification of two loci in tomato reveals distinct mechanisms for salt tolerance. *Plant Cell*, **13**, 873–887.

77 Borsani, O., Cuartero, J., Valpuesta, V. *et al.* (2002) Tomato tos1 mutation identifies a gene essential for osmotic tolerance and abscisic acid sensitivity. *Plant J.*, **32**, 905–914.

78 Rosado, A., Amaya, I., Valpuesta, V. *et al.* (2006) ABA- and ethylene-mediated responses in osmotically stressed tomato are regulated by the TSS2 and TOS1 loci. *J. Exp. Bot.*, **57**, 3327–3335.

79 Nunes-Nesi, A., Carrari, F., Gibon, Y. *et al.* (2007) Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. *Plant J.*, **50**, 1093–1106.

80 Hackel, A., Schauer, N., Carrari, F. *et al.* (2006) Sucrose transporter LeSUT1 and LeSUT2 inhibition affects tomato fruit development in different ways. *Plant J.*, **45**, 180–192.

81 Chen, T.H.H. and Murata, N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.*, **5**, 250–257.

82 Iordachescu, M. and Imai, R. (2008) Trehalose biosynthesis in response to abiotic stresses. *J. Integr. Plant Biol.*, **50** (10), 1223–1229.

83 Cortina, C. and Culiañez-Macia, F.A. (2005) Tomato abiotic stress enhanced

tolerance by trehalose biosynthesis. *Plant Sci.*, **169** (1), 75–82.

84 Garg, A.K., Kim, J.K., Owens, T.G. *et al.* (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc. Natl. Acad. Sci. USA*, **99**, 15898–15903.

85 Jang, I.C., Oh, S.J., Seo, J.S. *et al.* (2003) Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. *Plant Physiol.*, **131**, 516–524.

86 Karim, S., Aronsson, H., Ericson, H. *et al.* (2007) Improved drought tolerance without undesired side effects in transgenic plants producing trehalose. *Plant Mol. Biol.*, **64**, 371–386.

87 Miranda, J.A., Avonce, N., Suárez, R. *et al.* (2007) A bifunctional TPS-TPP enzyme from yeast confers tolerance to multiple and extreme abiotic-stress conditions in transgenic *Arabidopsis*. *Planta*, **226**, 1411–1421.

88 Moghaieb, R.E.A., Tanaka, N., Saneoka, H. *et al.* (2000) Expression of betaine aldehyde dehydrogenase gene in transgenic tomato hairy roots leads to the accumulation of glycine betaine and contributes to the maintenance of the osmotic potential under salt stress *Soil Sci. and Plant Nut.*, **46**, 873–883.

89 Han, S.E., Park, S.R., Kwon, H.B. *et al.* (2005) Genetic engineering of drought-resistant tobacco plants by introducing the trehalose phosphorylase (TP) gene from *Pleurotus sajor-caju*. *Plant Cell Tissue Organ Cult.*, **82**, 151–158.

90 Jia, G.X., Zhu, Z.Q., Chang, F.Q. *et al.* (2002) Transformation of tomato with the BADH gene from Atriplex improves salt tolerance. *Plant Cell Rep.*, **21**, 141–146.

91 Park, E.J., Jeknic, Z., Pino, M.T. *et al.* (2007) Glycinebetaine accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic tomato plants against abiotic stress. *Plant Cell Environ.*, **30**, 994–1005.

92 Sakamoto, A., Alia, Murata, N. (1998) Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. *Plant Mol. Biol.*, **38**, 1011–1019.

93 Wang, W., Vinocur, B., and Altman, A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, **218**, 1–14.

94 Zhang, Y., Wang, Z., and Xu, J. (2007) Molecular mechanism of dehydrin in response to environmental stress in plant. *Prog. Nat. Sci.*, **17**, 237–246.

95 Covarrubias, A.A. and Reyes, J.L. (2009) Post-transcriptional gene regulation of salinity and drought responses by plant microRNAs. *Plant Cell Environ.* doi: 10.1111/j.1365-3040.2009.02048.x.

96 Arrillaga, I., Gil-Mascarell, R., Gisbert, C. *et al.* (1998) Expression of the yeast HAL2 gene in tomato increases the *in vitro* salt tolerance of transgenic progenies. *Plant Sci.*, **136**, 219–226.

97 Mohamed, E.A., Iwaki, T., Munir, I. *et al.* (2003) Overexpression of bacterial catalase in tomato leaf chloroplasts enhances photo-oxidative stress tolerance. *Plant Cell Environ.*, **26**, 2037–2046.

98 Zhao, F., Guo, S., Zhang, H. *et al.* (2006) Expression of yeast SOD2 in transgenic rice results in increased salt tolerance. *Plant Sci.*, **170**, 216–224.

99 Tseng, M.J., Liu, C.W., and Yiu, J.C. (2007) Enhanced tolerance to sulfur dioxide and salt stress of transgenic Chinese cabbage plants expressing both superoxide dismutase and catalase in chloroplasts. *Plant Physiol. Biochem.*, **45**, 822–833.

100 Lee, Y.P., Kim, S.H., Bang, J.W. *et al.* (2007) Enhanced tolerance to oxidative stress in transgenic tobacco plants expressing three antioxidant enzymes in chloroplasts. *Plant Cell Rep.*, **26**, 591–598.

101 Yamaguchi, T. and Blumwald, E. (2005) Developing salt-tolerant crop plants: challenges and opportunities. *Trends Plant Sci.*, **10**, 615–620.

102 Chinnusamy, V., Jagendorf, A., and Zhu, J.K. (2005) Understanding and improving salt tolerance in plants. *Crop. Sci.*, **45**, 437–448.

103 Forrest, K.L. and Bhave, M. (2007) Major intrinsic proteins (MIPs) in plants: a

complex gene family with major impacts on plant phenotype. *Funct. Integr. Genomics.*, **7**, 263–289.

104 Gisbert, C., Rus, A.M., Bolarin, M.C. *et al.* (2000) The yeast *HAL1* gene improves salt tolerance of transgenic tomato. *Plant Physiol.*, **123**, 393–402.

105 Rus, A.M., Estañ, M.T., Gisbert, C. *et al.* (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. *Ann. Bot.* **103**: 609–623.

106 Gaxiola, R.A., Palmgren, M.G., and Schumacher, K. (2007) Plant proton pumps. *FEBS Lett.*, **581**, 2204–2214.

107 Apse, M.P., Aharon, G.S., Snedden, W.A. *et al.* (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis thaliana*. *Science*, **285**, 1256–1258.

108 Zhang, H.X., Hodson, J.N., Williams, J.P. *et al.* (2001) Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci. USA*, **98**, 12832–12836.

109 Zhang, H.X. and Blumwald, E. (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat. Biotechnol.*, **19**, 765–768.

110 Flowers, T.J. (2004) Improving crop salt tolerance. *J. Exp. Bot.*, **55**, 307–319.

111 Leidi, E.O., Barragán, V., Rubio, L. *et al.* (2010) The AtNHX1 exchanger mediates potassium compartmentation in vacuoles of transgenic tomato. *Plant J.*, **61**, 495–506.

112 Yokoi, S., Quintero, F.J., Cubero, B. *et al.* (2002) Differential expression and function of *Arabidopsis thaliana NHX* Na^+/H^+ antiporters in the salt stress response. *Plant J.*, **30**, 529–539.

113 Aharon, G.S., Apse, M.P., Duan, S. *et al.* (2003) Characterization of a family of vacuolar Na^+/H^+ antiporters in *Arabidopsis thaliana*. *Plant Soil*, **253**, 245–256.

114 Wang, W.Q., Li, Y., Zhang, Y.Y. *et al.* (2007) Comparative expression analysis of three genes from the *Arabidopsis* vacuolar Na^+/H^+ antiporter (AtNHX) family in relation to abiotic stresses. *Chinese Sci. Bull.*, **52**, 1754–1763.

115 Liu, H., Wang, Q.Q., Yu, M.M. *et al.* (2008) Transgenic salt-tolerant sugar beet (*Beta vulgaris* L.) constitutively expressing an *Arabidopsis thaliana* vacuolar Na^+/H^+ antiporter gene, AtNHX3, accumulates more soluble sugar but less salt in storage roots. *Plant Cell Environ.*, **31**, 1325–1334.

116 Gaxiola, R.A., Li, J.L., Undurraga, S. *et al.* (2001) Drought and salt tolerant plants result from overexpression of the AVP1 H^+ pump. *Proc. Natl. Acad. Sci. USA*, **98**, 11444–11449.

117 Park, S., Li, J., Pittman, J.F. *et al.* (2005) Up-regulation of a H^+-pyrophosphatase (H^+-PPase) as a strategy to engineer drought-resistant crop plants. *Proc. Natl. Acad. Sci. USA*, **102** (52), 18830–18835.

118 Gao, X.H., Ren, Z.H., Zhao, Y.X. *et al.* (2003) Overexpression of SOD2 increases salt tolerance of *Arabidopsis*. *Plant Physiol.*, **133**, 1873–1881.

119 Shi, H.Z., Lee, B.H., Wu, S.J. *et al.* (2003) Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana *Nat. Biotech.*, **21**, 81–85.

120 Wu, Y.Y., Chen, Q.J., Chen, M. *et al.* (2005) Salt-tolerant transgenic perennial ryegrass (*Lolium perenne* L.) obtained by *Agrobacterium tumefaciens*-mediated transformation of the vacuolar Na^+/H^+ antiporter gene. *Plant Sci.*, **169**, 65–73.

121 Martínez-Atienza, J., Jiang, X., Garciadeblas, B. *et al.* (2007) Conservation of the salt overly sensitive pathway in rice. *Plant Physiol.*, **143**, 1001–1012.

122 Kaur, N. and Gupta, A.K. (2005) Signal transduction pathways under abiotic stresses in plants. *Curr. Sci.*, **88**, 1771–1780.

123 Agarwal, P.K., Agarwal, P., Reddy, M.K. *et al.* (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Rep.*, **25**, 1263–1274.

124 Ham, B.K., Park, J.M., Lee, S.B. *et al.* (2006) Tobacco Tsip1, a DnaJ-Type Zn finger protein, is recruited to and potentiates Tsi1-mediated transcriptional activation. *Plant Cell*, **18**, 2005–2020.

125 Sohn, K.H., Lee, S.C., Jung, H.W. *et al.* (2006) Expression and functional roles of the pepper pathogen-induced transcription factor RAV1 in bacterial disease resistance, and drought and salt stress tolerance. *Plant Mol. Biol.*, **61**, 897–915.

126 Ogawa, D., Yamaguchi, K., and Nishiuchi, T. (2007) High-level overexpression of the *Arabidopsis* HsfA2 gene confers not only increased thermotolerance but also salt/osmotic stress tolerance and enhanced callus growth. *J. Exp. Bot.*, **58**, 3373–3383.

127 Liu, K., Wang, L., Xu, Y. *et al.* (2007a) Overexpression of OsCOIN, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice. *Planta*, **226**, 1007–1016.

128 Liu, N., Zhong, N.Q., Wang, G.L. *et al.* (2007b) Cloning and functional characterization of PpDBF1 gene encoding a DRE-binding transcription factor from *Physcomitrella patens*. *Planta*, **226**, 827–838.

129 Dai, X., Xu, Y., Ma, Q. *et al.* (2007) Overexpression of an R1R2R3 MYB gene, OsMYB3R-2, increases tolerance to freezing, drought, and salt stress in transgenic *Arabidopsis*. *Plant Physiol.*, **143**, 1739–1751.

130 Nakashima, K., Tran, L.S.P., Nguyen, D.V. *et al.* (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. *Plant J.*, **51**, 617–630.

131 Gu, Y.Q., Wildermuth, M.C., Chakravarthy, S. *et al.* (2002) Tomato transcription factors Pti4, Pti5, and Pti6 activate defense responses when expressed in *Arabidopsis*. *Plant Cell*, **14**, 817–831.

132 Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J. *et al.* (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell*, **15**, 165–178.

133 Huang, Z.J., Zhang, Z.J., Zhang, H. *et al.* (2004) Tomato TERF1 modulates ethylene response and enhances osmotic stress tolerance by activating expression of downstream genes. *FEBS Lett.*, **573** (1–3), 110–116.

134 Seong, E.S., Cho, H.S., Choi, D. *et al.* (2007) Tomato plants overexpressing CaKR1 enhanced tolerance to salt and oxidative stress. *Biochem. Biophys. Res. Commun.*, **363**, 983–988.

135 Vannini, C., Campa, M., Iriti, M. *et al.* (2007) Evaluation of transgenic tomato plants ectopically expressing the rice *Osmyb4* gene. *Plant Sci.*, **173**, 231–239.

136 Cheong, Y.H., Kim, K.N., Pandey, G.K. *et al.* (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. *Plant Cell*, **15**, 1833–1845.

137 Vashisht, A.A. and Tuteja, N. (2006) Stress responsive DEAD-box helicases: a new pathway to engineer plant stress tolerance. *J. Photochem. Photobiol. B*, **84** (2), 150–160.

138 Owttrim, G.W. (2006) RNA helicase and abiotic stress. *Nucleic Acids Res.*, **34**, 3220–3230.

139 Phillips, J.R., Dalmay, T., and Bartels, D. (2007) The role of small RNAs in abiotic stress. *FEBS Lett.*, **581**, 3592–3597.

140 Sunkar, R., Chinnusamy, V., Zhu, J. *et al.* (2007) Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends Plant Sci.*, **12** (7), 301–309.

141 Moller, I.S. and Tester, M. (2007) Salinity tolerance of *Arabidopsis*: a good model for cereals? *Trends Plant Sci.*, **12**, 534–540.

142 Cano, E.A., Perez-Alfocea, F., Moreno, V. *et al.* (1996) Responses to NaCl stress of cultivated and wild species and their hybrids in callus culture. *Plant Cell Rep.*, **15**, 791–794.

143 Pineda, B. (2005) Análisis functional de diversos genes relacionados con la tolerancia a la salinidad y el estrés hídrico en plantas transgénicas de tomate (*Lycopersicon esculentum* Mill). PhD Thesis, Universidad Politécnica de Valencia, Valencia, Spain.

144 Grover, A., Aggarwal, P.K., Kapoor, A. *et al.* (2003) Addressing abiotic stresses in agriculture through transgenic technology. *Curr. Sci.*, **84**, 355–367.

145 Romero, C., Belles, J.M., Vaya, J.L. *et al.* (1997) Expression of the yeast trehalose-6-

phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta*, **201**, 293–297.

146 Capell, T., Escobar, C., Lui, H. *et al.* (1998) Overexpression of the oat arginine decarboxylase cDNA in transgenic rice (*Oryza sativa* L.) affects normal development patterns *in vitro* and results in putrescine accumulation in transgenic plants. *Theor. Appl. Genet.*, **97**, 246–254.

147 Kasuga, M., Liu, Q., Miura, S. *et al.* (1999) Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.*, **17**, 287–291.

148 Rohila, J.S., Jainb, R.K., and Wu, R. (2002) Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley Hva1 cDNA. *Plant Sci.*, **163**, 525–532.

149 Su, J. and Wu, R. (2004) Stress-inducible synthesis of proline in transgenic rice confers faster growth under stress conditions than that with constitutive synthesis. *Plant Sci.*, **166**, 941–948.

150 Hsieh, T.H., Lee, J.T., Charng, Y.Y. *et al.* (2002a) Tomato plants ectopically expressing *Arabidopsis* CBF1 show enhanced resistance to water deficit stress. *Plant Physiol.*, **130**, 618–626.

151 Hsieh, T.H., Lee, J.T., Yang, P.T. *et al.* (2002b) Heterologous expression of the *Arabidopsis* CBF1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. *Plant Physiol.*, **129**, 1086–1094.

152 Lee, J.T., Prasad, V., Yang, P.T. *et al.* (2003) Expression of *Arabidopsis* CBF1 regulated by an ABA/stress inducible promoter in transgenic tomato confers stress tolerance without affecting yield. *Plant Cell Environ.*, **26**, 1181–1190.

153 Abebe, T., Guenzi, A.C., Martin, B. *et al.* (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. *Plant Physiol.*, **131**, 1748–1755.

154 Cherian, S., Reddy, M.P., and Ferreira, R.B. (2006) Transgenic plants with improved dehydration-stress tolerance: progress and future prospects. *Biol. Plant.*, **50**, 481–495.

155 Bhatnagar-Mathur, P., Vadez, V., and Sharma, K.K. (2008) Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. *Plant Cell Rep.*, **27**, 411–424.

156 Bohnert, H.J., Golldack, D., Ishitani, M. *et al.* (1996) Salt tolerance engineering requires multiple gene transfer, in *Engineering Plants for Commercial Products and Application* (eds G.B. Collins and R.J. Shepherd), New York Academy of Sciences.

157 Ray, S., Dansana, P.K., Bhaskar, A. *et al.* (2009) Emerging trends in functional genomic for stress tolerance in crop plants, in *Plant Stress Biology: From Genomics to Systems Biology* (ed. H. Hirt), Wiley Blackwell, Germany, pp. 37–63.

158 Sreenivasulu, N., Sopory, S.K., and Kavi Kishor, P.B. (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. *Gene*, **388**, 1–13.

159 Gruber, V., Blanchet, S., Diet, A. *et al.* (2009) Identification of transcription factors involved in root apex responses to salt stress in *Medicago truncatula*. *Mol. Genet. Genomics*, **281** (1), 55–66.

160 Wei, J., Tirajoh, A., Effendy, J. *et al.* (2000) Characterization of salt-induced changes in gene expression in tomato (*Lycopersicon esculentum*) roots and the role played by abscisic acid. *Plant Sci.*, **159**, 135–148.

161 Zhou, S.F., Chen, X.Y., Xue, X.N. *et al.* (2007) Physiological and growth responses of tomato progenies harboring the betaine alhyde dehydrogenase gene to salt stress. *J. Integr. Plant. Biol.*, **49**, 628–637.

162 Vicente Agulló, F.M., Garcia Abellán, J.O., Campos, J.F. *et al.* (2009) Differences in the regulation of salt tolerance between the cultivated tomato and its wild salt-tolerant species *Solanum pennellii* using microarray analysis. International Conference 'Plant Abiotic Stress Tolerance' Vienna (Austria), p. 149.

163 Bohnert, H.J., Gong, Q., Li, P. *et al.* (2006) Unraveling abiotic stress tolerance mechanisms: getting genomics going. *Curr. Opin. Plant Biol.*, **9**, 180–188.

164 Salekdeh, G.H., Siopongco, J., Wade, L.J. *et al.* (2002) A proteomic approach to analyzing drought- and salt-responsiveness in rice. *Field Crops Res.*, **76** (2–3), 199–219.

165 Khan, P.S.S.V., Hoffmann, L., Renaut, J. *et al.* (2007) Current initiatives in proteomics for the analysis of plant salt tolerance. *Current Sci.*, **93** (6), 807–817.

166 Jin, S., Chen, C.S., and Plant, A.L. (2000) Regulation by ABA of osmotic-stress-induced changes in protein synthesis in tomato roots. *Plant Cell Environ.*, **23**, 51–60.

167 Chen, C.S. and Plant, A.L. (1999) Salt-induced protein synthesis in tomato roots: the role of ABA. *J. Exp. Bot.*, **50**, 677–687.

168 Chen, S.B., Gollop, N., and Heuer, B. (2009) Proteomic analysis of salt-stressed tomato (*Solanum lycopersicum*) seedlings: effect of genotype and exogenous application of glycinebetaine, *J. Exp. Bot.*, **60** (7), 2005–2019.

169 Gygi, S.P., Rochon, Y., Franza, B.R. *et al.* (1999) Correlation between protein and mRNA abundance in yeast. *Mol. Cell Biol.*, **19**, 1720–1730.

170 Mooney, B.P., Miernyk, J.A., Greenlief, C.M. *et al.* (2006) Using quantitative proteomics of *Arabidopsis* roots and leaves to predict metabolic activity. *Physiol. Plant.*, **128**, 237–250.

171 Jiang, Y., Yang, B., Harris, N.S. *et al.* (2007) Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots, *J. Exp. Bot.*, **58** (13), 3591–3607.

172 Salt, D.E., Baxter, I., and Lahner, B. (2008) Ionomics and the study of the plant ionome. *Annu. Rev. Plant Biol.*, **59**, 709–733.

173 Baulcombe, D. (2004) RNA silencing in plants. *Nature*, **431**, 356–363.

174 Herr, A.J., Jensen, M.B., Dalmay, T. *et al.* (2005) RNA polymerase IV directs silencing of endogenous DNA. *Science*, **308**, 118–120.

175 Miki, D. and Shimamoto, K. (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant Cell Physiol.*, **45**, 490–495.

176 Hilson, P., Allemeersch, J., Altmann, T. *et al.* (2004) Versatile gene-specific sequence tags for *Arabidopsis* functional genomics: transcript profiling and reverse genetics applications. *Genome Res.*, **14**, 2176–2189.

177 Xiong, L. and Zhu, J.K. (2002) Molecular and genetic aspects of plant responses to osmotic stress. *Plant Cell Environ.*, **25**, 131–139.

178 Emmanuel, E. and Levy, A.A. (2002) Tomato mutants as genomic tools. *Curr. Opin. Plant Biol.*, **5**, 112–117.

179 Tanksley, S.D. (2004) The genetic, developmental, and molecular bases of fruit size and shape variation in tomato. *Plant Cell*, **16**, 181–189.

180 Giovannoni, J.J. (2007) Fruit ripening mutants yield insights into ripening control. *Curr. Opin. Plant Biol.*, **10**, 283–289.

181 Lozano, R., Giménez, E., Cara, B. *et al.* (2009) Genetic analysis of reproductive development in tomato. *Int. J. Dev. Biol.*, **53**, 1635–1648.

182 Zhu, J.K. (2000) Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiology*, **124**, 941–948.

183 Wu, S.J., Ding, L., and Zhu, J.K. (1996) SOS1, a genetic locus essential for salt tolerance and potassium acquisition. *Plant Cell.*, **8**, 617–627.

184 Liu, J. and Zhu, J.K. (1997) An *Arabidopsis* mutant that requires increased calcium for potassium nutrition and salt tolerance. *Proc. Natl. Acad. Sci. USA*, **94**, 14960–14964.

185 Zhu, J.K., Liu, J., and Xiong, L. (1998) Genetic analysis of salt tolerance in *Arabidopsis*. Evidence for a critical role of potassium nutrition. *Plant Cell*, **10**, 1181–1191.

186 Shi, H., Ishitani, M., Wu, S.J. *et al.* (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. *Proc. Natl. Acad. Sci. USA*, **97**, 6896–6901.

187 Shi, H., Quintero, F.J., Pardo, J.M. *et al.* (2002) The putative plasma membrane Na1/H1 antiporter SOS1 controls long-distance Na1 transport in plants. *Plant Cell*, **14**, 465–477.

188 Quesada, V., Ponce, M.R., and Micol, J.L. (2000) Genetic analysis of salt-tolerant mutants in *Arabidopsis thaliana*. *Genetics*, **154** (1), 421–436.

189 Bohnert, H., Ayoubi, P., Borchert, C. *et al.* (2001) A genomics approach towards salt stress tolerance. *Plant Physiol. Biochem.*, **39**, 295–311.

190 Kumar, S., Allen, G.C., and Thompson, W.F. (2006) Gene targeting in plants: fingers on the move. *Trends Plant Sci.*, **11** (4), 159–208.

191 Comai, L. and Henikoff, S. (2006) TILLING: practical single-nucleotide mutation discovery. *Plant J.*, **45** (4), 684–694.

192 McCallum, C.M., Comai, L., Greene, E.A., and Henikoff, S. (2000) Targeted screening for induced mutations. *Nat. Biotechnol.*, **18** (4), 455–457.

193 Colbert, T., Till, B.J., Tompa, R., Reynolds, S. *et al* (2001) High-throughput screening for induced point mutations. *Plant Physiol.*, **126** (2), 480–484.

194 Triques, K., Piednoir, E., Dalmais, M. *et al.* (2008) Mutation detection using ENDO1: application to disease diagnostics in humans and TILLING and Eco-TILLING in plants. *BMC Mol. Biol.*, **9**, 42.

195 Gady, A.L.F., Hermans, F.W.K., Van de Wal, M.H.B.J., van Loo, E.N., Visser, R.G.F., and Bachem, C.W.B. (2009) Implementation of two high through-put techniques in a novel application: detecting point mutations in large EMS mutated plant populations. *BMC Plant Method*, **5**, 13.

196 Krysan, P.J., Young, J.C., and Sussman, M.R. (1999) T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell*, **11**, 2283–2290.

197 Springer, P.S. (2000) Gene traps: tools for plant development and genomics. *Plant Cell*, **12**, 1007–1020.

198 Antón, T., García-Abellán, J.O., Pérez, F. *et al.* (2009) Identification of genes related to salt and drought tolerance in T-DNA tagging lines of cultivated and wild tomato species. International Conference 'Plant Abiotic Stress Tolerance' Vienna (Austria), p. 161.

199 Barrero-Gil, J., Rodríguez-Navarro, A., and Benito, B. (2007) Cloning of the PpNHAD1 transporter of *Physcomitrella patens*, a chloroplast transporter highly conserved in photosynthetic eukaryotic organisms. *J. Exp. Bot.*, **58**, 2839–2849.

200 Zhu, J.H., Verslues, P.E., Zheng, X.W. *et al.* (2005) HOS10 encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants. *PNAS*, **102** (28), 9966–9971.

201 Jung, C., Seo, J.S., Han, S.W. *et al.* (2008) Overexpression of AtMYB44 enhances stomatal closure to confer abiotic stress tolerance in transgenic *Arabidopsis*. *Plant Physiol.*, **146** (2), 623–635.

202 Gao, L. and Xiang, C.B. (2008) The genetic locus At1g73660 encodes a putative MAPKKK and negatively regulates salt tolerance in *Arabidopsis*. *Plant Mol. Biol.*, **67**, 125–134.

203 Casson, S. and Gray, J.E. (2008) Influence of environmental factors on stomatal development. *New Phytol.*, **178**, 9–23.

44
Potato: Improving Crop Productivity and Abiotic Stress Tolerance

Gefu Wang-Pruski and Andrew Schofield

Potato is a prestige food crop that billions of people in the world depend on as an energy source. Its widespread cultivation and adaptation have rendered it attractive for humans of all cultures and geographic locations. However, its effective production is challenged by many unfavorable environmental conditions, such as drought, heat, cold, and salinity. Recent tools developed in genomic research have advanced our understanding of the potato crop in its ability to manage these stresses. This chapter provides the latest information on our understanding of stress tolerance mechanisms in potatoes based on functional genomics, transcriptomics, proteomics, and metabolomics studies. We also recommend many choices of functional genes that can be used for improving stress tolerance in potatoes. We would like to point out that due to lack of the completion of the potato genome sequence, many research projects are not able to provide more advanced systems biology data in comparison to that available for many other agricultural species.

44.1
Introduction

The potato crop (*Solanum tuberosum* L.) is ranked as the fourth food crop in the world after rice, wheat, and maize (FAOSTAT). Its production in 2009 reached 329.6 million ton with China as the top producer with 69 million ton, followed by India with 34 million ton. A detailed, country-wise information on potato production, utilization, and trade can be found in the annually updated databases of the United Nations Food and Agriculture Organization (FAO). The potato is a starchy, tuberous crop from the Solanaceae family known as the nightshades. It originated in the region of the Andes and was introduced to the rest of the world four centuries ago. Today, potatoes have become an integral part of much of the world cuisine due to their rich content of carbohydrates, proteins, vitamin C, iron, and fiber.

Potatoes are grown in about 100 countries, occupying every continent of the world. In some parts of the world, true botanical seeds are used to produce the crop, although most of the North American and European production systems rely on tuber seeds because of their genetic uniformity for traits such as size, color, texture,

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and biochemical contents. Potatoes are used as fresh, processed, and value-added food products, as well as a source of industrial starch. The variety requirements depend on needs, including tuber shape and size, sugar content, frying color, nutritional value, storability, and disease resistance. The issues facing the industry are high acrylamide content in processed products, obesity related to consumption, and sustainable production systems for disease and pest control.

Nitrogen source is essential for tuber production. Nitrogen available to plants in the soil and nitrogen loss in the system determine how much nitrogen is actually used by the plants. Because excess water can lead to nutrient leaching, water becomes a key factor in determining how nitrogen can be taken up by plants. Estimation of irrigation needs have been established in many countries based on the variety and climate conditions. As precision farming for crop management increased in popularity, effective nitrogen and water applications may be found in potato production systems around the world. Nevertheless, severe weather conditions and climate change will continue to challenge the potato production systems in different parts of the world. Therefore, generation of new cultivars adapted to different stress conditions will become essential for potato production in the coming years.

44.2 Potato Genomic Resources

Besides the cultivated potato (*S. tuberosum* ssp. *tuberosum*), other crops such as tomato, pepper, eggplant, and tobacco are also key members of the Solanaceae family. Their genetic information is important for understanding the genome of potatoes. For instance, tomato (*S. lycopersium*) has a similar genome size to potato and is seen as a genetic and genomic model for the Solanaceae family. Cultivated potato behaves as an autotetraploid and has $2n = 4x = 48$ chromosomes. It is generally understood that potato has a genome size of 850–1000 Mb, which is very similar to that of tomato. However, a large number of wild and hybrid diploid selections are being used for genetic mapping-related studies due to their reduced complexity of genome recombination. Nevertheless, the lack of homozygous lines in potatoes makes gene mapping and breeding a very slow and challenging process.

The potato genome sequencing was projected to be completed within 6 months by the international Potato Genome Sequencing Consortium (PGSC). The completion of the genome sequence will no doubt provide valuable information about the genome arrangement, diversity, functional genes, and gene alleles. Major potato genome databases available are PGSC (www.potatogenome.net), The Institute for Genomic Research (TIGR) Solanaceae Genomics Resource (http://jcvi.org/potato), The Canadian Potato Genome Project (www.cpgp.ca), Solanaceae Coordinated Agricultural Project (http://solcap.msu.edu), and the DFCI *Solanum tuberosum* Gene Index (StGI) (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb = potato). Many functional genomics studies have already used the sequencing information for trait analysis and marker discovery. Over the past 10 years, the potato research community around the world has also established extensive genomic

resources, including expressed sequence tag (EST) libraries, SAGE libraries, microarrays, molecular function maps, and mutant populations [1–3]. Genomic data from EST libraries, molecular linkage maps, and microarray expression analyses will help identify the genetic components underlying many of the traits, for example, yield, quality, and abiotic and biotic stress managements. In addition, natural genetic resources (wild potato varieties and germplasm collections) can be used in concert with genomic tools such as marker-assisted selection, polymorphism identification, and association mapping to improve breeding lines.

44.3
Abiotic Stresses Related to Potato Production

Potatoes grow the best under long and hot day and cool night conditions. They are very sensitive to light, water, and sources of nitrogen. Any stress condition, such as water, temperature, salinity, or mechanical damage, will significantly impact the yield, tuber quality, and marketing value. Even a short period of acute stress can cause a substantial decrease in total and marketable yield. For the North American market, 50% of the potatoes are processed during storage, so postharvest stress to tubers can further reduce the marketable quality and produce low temperature-induced cold sweetening and after-cooking darkening (ACD), disorders that are induced or exacerbated by storage conditions.

This chapter will focus on five key abiotic stress issues: drought, heat, cold and frost, salinity, and wounding. Since the molecular basis for the stress responses is the key to understanding the stress management mechanisms in potatoes, we have provided information about cellular responses and regulation of each stress, and then provided an overview on the research outcomes in molecular gene functions and omics studies. Owing to the large volume of literature for these topics in potatoes, we did not compile an exhaustive list of references, but rather included those that highlight key findings and employ an "omics" perspective to provide insight to direct future work.

44.3.1
Water and Drought Stress

Water is becoming an increasingly scarce resource on earth. Approximately, 80 countries with 40% of the world population suffer from serious water shortage. The potato produces more food energy per unit water on dry weight basis than the other food crops [4]. Thus, water productivity in potatoes is two to three times higher than that of maize, rice, and wheat [5]. Ironically, although the potato crop uses water relatively efficiently, it is also characterized as more drought sensitive than other agronomic crops. This is, in part, due to a lower root length density compared to the other crops. Therefore, in the low rainfall areas, utilization of irrigation systems is not uncommon if higher yield and quality are desired. Drought tolerance is measured by the relative ability of a variety to produce tubers from a limited amount of water [6]. However, water requirements vary for different stages of plant development (shoot

and stem growth, and tuber formation) and tuber bulking, making the entire process difficult to control and predict. Studies normally look into early drought or late drought conditions. If the drought conditions appear early in the season, the plant's survival strategy is to minimize water losses to transpiration; thus, leaf growth begins to slow down, followed by reducing areal growth and canopy size [7]. If the conditions do not improve, the plants will have a reduced capacity for light interception, resulting in lower yields [8].

In potato production, varieties are often categorized by early or late maturing. This makes the situation even more difficult to manage. Some early-maturing potato varieties escape late-season drought events, whereas late-season drought may significantly impact yield losses in late-maturing varieties [9]. There is a variety difference in drought tolerance in potatoes and the effect of drought timing also depends on genotype. For some varieties, the effect is more profound when drought occurs during tuber initiation [10–12], while in others more critical during tuber bulking period [13, 14].

44.3.1.1 Effect of Drought on Tuber Quality

Drought has significant impact on a variety of tuber qualities and defects. Drought can cause tuber cracking, secondary growth, malformations, hollow heart, and internal brown spot. Drought can adversely increase the contents of glycoalkaloids such as α-solanine and α-chaconine, compounds that are believed to cause cancer and other health problems. Drought can also cause sugar end, a disorder characterized by relatively low starch and high sugar content in the basal end of the tuber. Processing these tubers resulted in French fries with dark and discolored ends. Stressed plants accumulate large amounts of sucrose in the basal tissues of the tuber immediately following stress. This is because water deficit, as well as heat stress, induces changes in the activities of certain key carbohydrate metabolizing enzymes, shifting the tuber from a starch synthesizing function to starch mobilization. Drought-stressed plants are also more susceptible to biotic stresses; for example, drought conditions increase pest disease infestations of cyst nematodes [15, 16] and drought reduces transpiration and stomatal conductance, which increases *Verticillium*-related wilting disease.

44.3.1.2 Drought Sensing Mechanisms

Root dry mass was found to be critical for water stress conditions, as it is significantly correlated with leaf area, photosynthesis, reduction of stomatal conductance, and tuber yield [17]. If drought occurs before tuberization, the plants will lose the ability to produce a higher number of stolons per stem [18], resulting in a lower tuber yield. If it is during tuber bulking stage, it will lower both tuber number and size [19]. It is understood that plant responses to drought, including stomatal reactions, are triggered by root signals, not just leaf water potential. In this case, abscisic acid (ABA) plays an essential role in stress signaling. At least four independent regulatory systems for gene expression changes in response to water stress have been identified, two are ABA dependent and two are ABA independent [20]. When potato roots sense soil water deficits, well before leaf water potential drops, ABA is produced in root tips

and transported through the xylem to the leaves to attenuate growth and close stomata [21]. Such signal transduction pathways include a plethora of secondary messengers such as hormones, phospholipids, and calcium ions [22].

Decreased accumulation of reactive oxygen species (ROS), greater mitochondrial activity, and active chloroplast defenses all contributed to the management of drought stress in a drought-resistant cultivar. In stressed plants, the primary sources of ROS are the Mehler reaction and the antenna pigments in the chloroplasts, the photorespiratory pathway in peroxisomes, the cytochrome reactions in the endoplasmic reticulum and cytoplasm, and oxidative processes in the mitochondria. Under drought conditions, drought-tolerant potato lines upregulated members of all major ROS scavenging enzymes in the chloroplasts: superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase, and peroxiredoxin gene families [23–26]. In addition, other genes encoding proteins that contribute to increased ROS scavenging capacity such as glutathione synthetase, glutathione-*S*-transferase, glutathione transporter, two thioredoxins, and four thioredoxin-related chloroplast-targeted genes were induced in a drought-resistant accession [24, 27]. Evidence also showed that several genes in the biosynthesis pathway of antioxidant compounds, such as flavonoids, anthocyanins, and xanthophylls, were strongly induced in tolerant cultivars. In another study, gene transcripts of three key enzymes in the biosynthesis of flavonoid and carotenoids were increased in leaves and roots under mild and moderate drought conditions [28].

Osmotic adjustment is the process by which plant cells maintain turgor during water deficit. The osmotic potential inside the cytoplasm is lowered by the accumulation of osmolytes (also called compatible solutes) such as amino acids and sugars [29]. Proline and trigonelline are two compounds that have received a lot of attention as potential compatible solutes and it has been suggested that proline could act as an antioxidant [30]. The accumulation of these compounds is often observed in response to hyperosmotic stress [31, 32] and in response to a combined treatment of heat and drought stress [33, 34]. In one study, drought caused an increase in proline, trigonelline, and proline analogues in a drought-resistant potato cultivar, whereas the drought-susceptible cultivar had an increase only in proline analogues [35]. In another study, proline increased in both drought-resistant and -susceptible potato cultivars [24]. Nevertheless, the role of proline remains controversial. Proline levels often increase earlier in drought-susceptible varieties than in more tolerant ones, which has led to the conclusion that proline is only an indicator of plant water status but not of tolerance. Others have suggested that because minor proline analogues, such as hydroxyproline, increase during drought stress, they may play a role in increased synthesis or reconstitution of cell wall components [36], and there is some evidence that free proline synthesized in other plant parts is transported to the roots to be used for cell wall synthesis in the apical region [37].

44.3.1.3 **Breeding through Omics Approaches**

Traditional breeding has made limited success in drought tolerance using existing potato germplasm. This is partly because the timing and severity of naturally occurring drought is quite erratic and so plants tend to respond differently from

year to year. As a result, the early stages of breeding programs often aim for high yield in favorable environments, but these gains are not usually maintained in environments commonly affected by severe stress. In addition, direct selection for drought tolerance under water-stressed conditions is hampered by low heritability, polygenic control, and epistasis of many drought tolerance traits [27]. Another obstacle for breeding programs is a reluctance to use unadapted parents, such as a wild potato species. It is also difficult to breed for tropical environments where high temperatures are a factor because many drought tolerance traits might not be effective when the plant experiences a combination of both drought and heat effects.

44.3.1.4 **Pathways Involved in Drought Stress**

Comparing transcriptomic and metabolomic profiles of tolerant and susceptible genotypes has led to the identification of some changes in photosynthesis and carbon and amino acid metabolism that are closely related to drought tolerance [35]. It was also found that drought stress regulates osmotic adjustment, carbohydrate metabolism, membrane modifications, strengthening of cuticle and cell rescue mechanisms, detoxification of oxygen radicals, and protein stabilization [24]. In addition, drought stress increases ethylene biosynthesis that may subsequently increase stress perception since ethylene and ABA attenuate leaf growth under water stress [24, 38]. Drought treatment also induces a number of ABA-responsive genes and leads to an accumulation of gibberellins degrading enzymes.

Stomatal closure under drought conditions prevents CO_2 supply for photosynthesis, leading to a reduction in net photosynthesis. This repression triggers regulation of genes functioning in the light reaction, Calvin cycle, and chlorophyll biosynthesis [35] and is accompanied by an increased expression of genes related to photorespiration and cyclic electron transport in photosystem I [24, 35]. Some drought-resistant genotypes, presumably under tolerable drought conditions, upregulated photosynthesis-related genes, while the same genes are downregulated in drought-susceptible genotypes [39].

Drought stress also represses transcription of genes involved in carbohydrate biosynthesis, glycolysis, and the tricarboxylic acid cycle, while sucrose metabolism is induced. These changes are required because under drought conditions, carbohydrate metabolism is redirected to reserve mobilization, as illustrated by induction of starch degrading enzymes, invertase and sucrose synthase [23, 24]. This was proved by Watkinson's study [40] in which expression profiles of genes associated with carbon metabolism contributed to differences in tuber development in phenotypes of adapted and acclimated, drought-stressed *S. tuberosum* ssp. *andigena*.

Finally, attention needs to be given to genotype differences in stress response strategies. Mane *et al.* [23] analyzed two potato landraces during drought and drought recovery. One landrace, Sullu, maintained vegetative biomass accumulation during drought while the other, Ccompis, experienced reduction in vegetative growth. Interestingly enough, both landraces maintained the same tuber yields as nonstressed controls. In Sullu, the main response that helped maintain vegetative growth

appears to be a capacity to uphold photosynthetic efficiency, minimize stomatal resistance, and activate photosynthetic genes during recovery. Other differences that are perhaps caused by the increased photosynthetic capacity include increased cell wall biosynthesis, maintenance of plastid SOD transcripts, and significant increases in sucrose, trehalose, and proline. By contrast, Ccompis differs in all of these aspects, most importantly in respect to photosynthesis. This study demonstrates that a diversity of effective strategies for dealing with abiotic stresses may exist naturally within the potato germplasm.

44.3.1.5 Genes Involved in Drought Stress Signaling

Many genes have been identified and their functional studies have shown promises with regard to drought tolerance. Transcriptomic studies found upregulation of genes for Ca^{2+} binding and GTP binding factors, kinases, and phosphatases [24]. Genes encoding several protein phosphatases 2C (PP2C), negative regulators of ABA signaling in *Arabidopsis*, are potential candidates for yield maintenance under drought conditions [41, 42]. Drought stress induced nitrite reductase and a chloroplast PII nitrogen-sensing protein, known to activate glutamine synthase [43]. Cysteine biosynthesis and sulfur uptake genes, such as adenosine phosphosulfate reductase, are upregulated at an early stage of drought condition, but reversed to repression under prolonged drought [35].

Transcription factors are actively involved in drought stress response. Both ATHB-7 and RD26 are upregulated by drought and function in one of the ABA-dependent regulatory systems, whereas a dehydration-responsive element DREB is regulated in ABA-independent regulatory systems [39]. Two other factors, ASR1 and ASR2, are strongly upregulated by drought stress [24]. ASR1, when present in the nucleus, regulates the expression of a hexose transporter, while in the cytosol, it functions as a chaperone to stabilize proteins under abiotic stress conditions [44]. Other transcriptional factors found are members of the WRKY, SCARECROW, MYB, CCR-4, TAF-3, and NAM transcription factor families. They are commonly induced by elevated H_2O_2 levels in potato leaves under drought conditions [24].

Polyols are osmotically active solutes that can effectively replace water in establishing hydrogen bonds and thereby protect enzyme activities and membranes experiencing water stress [45]. Drought-stressed cv. Sullu had both increased levels of a polyol (galactinol) and its precursors (galactose and inositol) and increased transcript levels of two genes (glucose-4-epimerase and galactinol synthase) involved in galactinol synthesis [35], whose overexpression in *Arabidopsis thaliana* has been shown to increase drought tolerance [46, 47]. Transgenic potato lines of cv. Desiree that overexpressed a dehydrin 4 (DHN4) isolated from barley, or a stress-inducible, heat-stable LEA group 3-like protein from bromegrass (ROB5), showed significant potential to enhance yield under moisture stress [48]. Interestingly, a wild tuber-bearing species, *Solanum gandarillasii* Cardenas, was found to exhibit reduced osmotic adjustment responses resulting in low transpiration rates [49].

Membranes are the main targets of degenerating processes caused by drought. During drought stress, there is a significant upregulation of nonspecific lipid transfer protein genes [50]. Other drought stress-induced gene products, such as heat shock

proteins (HSPs) and chaperones are used to protect cellular structures by maintaining the hydration of cellular compounds such as proteins and membranes. One orthologue to the *Arabidopsis* HSP At5g12030 was found to enhance drought tolerance [51]. An HSP DnaJ gene family was found to increase drought tolerance in potatoes [24, 25]. Others reported that an ATP-dependent metalloprotease and chaperone are induced in potatoes for drought tolerance [52].

44.3.1.6 Gene Testing in Transgenic Lines

Many labs have employed a transgenic approach to test candidate genes for improved drought tolerance in potatoes. Waterer *et al.* [48] tested the functions of constitutive CaMV 35S promoter or a stress-induced *Arabidopsis* COR78 promoter for the overexpression of four transgenes. The transgenic lines with the COR78 promoter produced higher yield under nonstressed conditions than the 35S promoter [48]. Interestingly enough, most of the transgenic lines demonstrated higher yields under drought stress in field trials. As plants respond to many stresses using similar mechanisms, Tian *et al.* [53] studied the potato zinc finger protein StZFP1 that is induced by salt and exogenous ABA. Findings are worth to explore further for studying stress management in plants. Transgenic potatoes expressing trehalose-6-phosphate synthase (TPS1) had a 30–40% reduction in stomatal densities. This appeared to cause lower CO_2 fixation rates under normal and drought-stressed conditions, enabling TPS1 plants to conserve water [54].

Transgenics overexpressing genes encoding antioxidant enzymes improved the drought tolerance of potatoes. Overexpression of nucleoside dikinase 2, Cu/Zn-SOD, and APX improves drought tolerance [55–57]. Transgenic potato lines of cv. Desiree that overexpressed wheat mitochondrial MnSOD3:1 under the direction of a stress-inducible COR78 promoter showed significant potential to enhance yields under moisture stress [48]. Simultaneous expression of choline oxidase, superoxide dismutase, and APX in the potato chloroplasts provides synergistically enhanced protection against salt and drought stresses at the whole-plant level [58]. Transgenic potatoes overexpressing *Arabidopsis* glutathione reductase gene (AtGR1) exhibited faster recovery from drought and with less visual injury compared to nontransformed controls [59]. Higher percentages of the reduced ascorbate were observed for transgenic potato and poplar trees with overexpressed glutathione reductase [60], which might be attributed to the higher glutathione levels in the transgenic plants [59]. Another study showed that overexpression of both Cu/Zn-SOD and APX genes in sweet potato (*Ipomoea batatas*) leaves can protect them from stress environment and enhance their drought tolerance. Finally, it was found that genes related to the accumulation of ROS could improve drought, salinity, and oxidative stress tolerance [61].

44.4 Heat Stress and Thermotolerance

Temperature during the growing season affects the dynamics of the growth and development of the potato plants, resulting in the significant effects on yield and

quality of potato tubers. For a complete coverage of the issue, read the comprehensive review given by Struik [62]. As Striuk pointed out, it is important to note the parts of the plants that are exposed to certain temperatures and the period of exposure to specific temperature treatments. In this review, estimated optimal temperatures for each stage of the plant cycle are given. Temperatures significantly higher or lower than the optimal levels will cause stress to the plants or can damage the plant or tubers during growth and storage. Heat injury and sunscald are the damages to foliage and tubers caused by high temperature ($>25\,^{\circ}C$) and/or direct sunlight. Most of the leaf injury occurs during intense dry weather and when there is a strong wind (air temperature $>30\,^{\circ}C$). Tubers lying in the collection rows after digging may be injured internally or externally when exposed to direct solar radiation and high temperatures.

Recent attention on global warming should bring some concerns on potato production. Projections indicate that global average temperatures will increase from 1.1 to $6.4\,^{\circ}C$ by the end of this century, depending upon region [63]. Generally, increasing CO_2 concentrations and air temperatures will result in lower growth and yield, reductions in the duration of the plant cycle and increase in potato diseases. Thus, the development of cultivars that are tolerant to high temperatures is critical to the strategy to minimize the global warming effects. Furthermore, the problem is compounded when other stresses, such as drought and salt, are factored in. For example, some wild *Solanum* species effectively deal with drought stress by reducing transpiration rates; however, this also reduces evaporative cooling effects that in turn require leaves to possess a higher thermal tolerance [49]. If heat stress is added to salt stress 40–60 days after emergence, the mechanisms that normally prevent salt accumulation fail and the young expanding leaves can be permanently damaged.

A number of tools including cultural practices and genetics are available to ameliorate heat stress. It appears that potato leaves grown under heat stress exhibit impaired cell expansion, but this can be overcome by increasing root zone calcium levels to promote axillary shoot growth [64]. Literature has suggested a significant amount of variation in tolerance to heat and cold stresses both among *S. tuberosum* cultivars and wild relatives [23, 49, 65–69]. These variations may provide breeding materials to develop thermotolerant varieties.

44.4.1 Effect of Heat on Tuber Quality

Heat stress and water deficit appear to induce changes in carbohydrate metabolism in the tuber, shifting from starch synthesis to starch mobilization [70]. The frequency and severity of internal heat necrosis, a disorder that manifests in the form of brown spots in the tuber flesh, affects fresh market and processing quality. Such defect occurs when the early growing season is subjected to high day and night temperatures and low rainfall [71]. The same weather conditions can also cause sugar end, which results in dark colored French fries. In addition, skin russeting occurs in potato tubers exposed to high soil temperatures, which triggers the production of thick and protective skin layers that become cracked with subsequent tuber expansion [72].

44.4.2
Cellular Response to Heat

Heat as a signal triggers several plant responses, including production of HSPs, molecular chaperones, osmoprotectants, and oxidative response pathways. These molecules protect macromolecular structures and proteins from denaturation during heat stresses. For example, stress-induced tomato ABA stress ripening 1 (SIASR1) protein has chaperone-like activity and can stabilize a number of proteins against denaturation caused by heat and freeze–thaw cycles [44]. The heat-tolerant potato cultivar Norchip, when exposed to high temperatures (40 °C), synthesized small HSPs for a longer time period than other more heat-sensitive cultivars [69]. Oxidative stress also plays a role in heat stress. For example, a sweet potato peroxidase (SWPA4) responds to several abiotic stresses and contains a *cis*-acting heat shock element in its promoter [73]. Tang *et al.* [56, 74] developed several potato lines using an oxidative stress-inducible SWPA2 promoter to express either Cu/Zn SOD and APX in the chloroplasts or *Arabidopsis* nucleoside diphosphate kinase 2 (NDPK2) in the cytosol. In both cases, the presence of the transgene greatly diminished the reduction in photosynthetic activity that was caused by high temperatures (42 °C for 20 h) [56, 74]. In another study, Waterer *et al.* [48] transformed cultivar Desiree to overexpress one of the four genes: mitochondrial MnSOD3:1 from wheat or a cold-inducible transcriptional factor DREB/CBF1 from canola, as well as the two previously mentioned genes encoding dehydrin 4 (DHN4) from barley and LEA group 3-like (ROB5) from bromegrass [48]. All of the transgenes appeared to enhance the heat stress tolerance (44 °C) of whole plants or excised leaves, with lines transformed with SOD3.1 showing the greatest effect. In low-temperature stress trials conducted under controlled environment and in the field, lines overexpressing SOD3:1 showed an enhanced capacity to grow at suboptimal temperatures (10 °C), while lines transformed with SOD3.1 or ROB5 had greater tolerance to freezing temperatures than the parental lines [48]. Therefore, there may well be similar cellular mechanisms in potatoes when dealing with high or low temperature stresses.

44.4.3
Cultivar Development through Omics Approaches

Few studies have been completed at the omics levels. Below are the three individual studies that have provided some key information about the pathways that are regulated by heat, cold, and salt. Readers should by now realize that cells use similar mechanisms to respond to different stresses, that is why often similar genes and pathways are found under different stress stimuli. Rensink *et al.* [75] generated 20 756 ESTs from a cDNA library constructed by pooling mRNA from heat, cold, salt, and drought-stressed potato leaves and roots and termed it the potato abiotic stress (POA) collection. This collection contained 1476 unique sequences, 667 contigs, and 809 singleton ESTs. In their subsequent study [76], researchers used a ~12 000 clone

potato cDNA microarray to measure the expression of seedlings grown under controlled conditions and subjected to cold (4 °C), heat (35 °C), or salt (100 mM NaCl) stress for up to 27 h. They discovered that potato gene products implicated in stress adaptation were similar to those reported in other plant species, such as molecular chaperones, HSPs, late-embryogenesis abundant proteins, and gene products with enzymatic activity, as well as several transcription factors, signal transduction proteins, and hormone signaling-related genes. In a study that focused on skin russeting induced by heat stress, Ginzberg *et al.* [72] looked into the transcriptomic profile of the periderm of tubers. Results revealed the upregulation of genes encoding heat shock proteins and regulation of transcription factors and genes related to cell proliferation and differentiation.

44.5 Cold and Frost Stresses

Potato plants can be injured by low temperature and frost in the field during the growing season. Low-temperature injury occurs when the leaf temperature drops below 0 °C, but tissues are not yet frozen. Frost damage occurs when the leaf tissues become frozen. At the end of the growing season, tubers in the ground can be injured by cold and frost. They become very sensitive to mechanical damages due to lifting, transport, and storage. Potato tubers can be damaged when the temperature is below 3 °C. The severity of the damage depends on variety, temperature, and the exposure duration. When the storage temperature is between freezing and 9 °C, tuber starch is converted into sugar, resulting in cold sweetening. Cold sweetening is a major processing defect, causing brown color after frying for French fries and chips. When the temperature is below 3 °C, tubers can be damaged internally and externally. The damaged tissues will rot by bacterial pathogens soon after.

Cold conditions that could cause damage to potatoes are temperatures below which cells can handle for normal physiological activities. For clarity in this section, we will use the terms cold tolerance and cold acclimation to refer to low temperatures above freezing, and we will use freezing tolerance to refer to temperatures below 0 °C. While cultivated potato (*S. tuberosum*) does not have the ability to cold acclimate, some wild *Solanum* species can be acclimated to cold by exposure to low temperature (~4 °C) for a period of time [77]. The degree of cold acclimation can be assessed by several methods including visual inspection or by measuring changes in electrical conductivity of the leaves and estimated by the LT50 corresponding to the temperature inducing 50% of injured cells [77]. There is a quite overlap among different stress responses. For example, microarray analysis indicates that leaves respond to cold and salt stresses very similarly at 9 and 27 h [76].

In response to low-temperature stress, plant membrane lipids have a tendency to change from gel to liquid-crystalline phase due to the increased level of lipid desaturation. Thus, fatty acid desaturases have been the focus of improving cold tolerance in plants [78]. It has been found that an increase in 18 : 2 (linoleate) in the

purified plasma membrane fraction during cold acclimation is associated with genetic variations in cold acclimation capacity [79]. This increase was found only in genotypes that are able to cold acclimate and was reversible on deacclimation, suggesting a link between the accumulation of 18 : 2 and the acquisition of freezing tolerance [80]. An accumulation of Δ9 desaturase gene transcripts during cold acclimation is confirmed to be associated with the cold acclimation response in potatoes [80]. Also, cold tolerance (long-term growing at 8 °C), as well as freezing tolerance (−7 °C for 30 min), was enhanced in transgenic lines expressing an acyl-lipid Δ12-desaturase gene from *Synechocystis* spp. PCC6803 because of an increased unsaturated fatty acid concentration in their lipids, with increased content of 18 : 2 and 18 : 3 fatty acids [81, 82].

Lipid profile is, therefore, an effective measure of the cellular responses to cold stress. One study compared the lipid profiles between a freezing-tolerant, cold-acclimating wild potato species (*S. commersonii*) and a freezing-sensitive, nonacclimating cultivated species (*S. tuberosum*). Following cold acclimation, both species had a decrease in palmitic acid, an increase in unsaturated to saturated fatty acid ratio, an increase in free sterols, an increase in sitosterol, and a slight decrease in cerebrosides. Lipid changes detected only in the acclimating species included an increase in phosphatidylethanolamine, a decrease in sterol to phospholipid ratio, an increase in linoleic acid, a decrease in linolenic acid, and an increase in acylated steryl glycoside to steryl glycoside ratio [83]. This study highlighted the importance of membrane lipid profiles in regulating frost and cold tolerance in plants. Another study [84] has shown that during low-temperature treatment, *Solanum* species that are able to acclimate to cold induced a noticeable increase in SsLTP1 (a lipid transfer protein), while species that display a low capacity for cold acclimation had no change in SsLTP1 levels.

44.5.1 Cellular Response to Cold

In response to cold stress, cells accumulate osmoprotectants, small molecules that balance the osmotic difference between the cell surroundings and the cytosol. Upon cold exposure, carbohydrates accumulated in cultivars that are cold tolerant [77]. It is worth to point out that even under nonstressed conditions, most carbohydrates (sucrose, galactose, galactinol, raffinose, and glucose) were present at higher levels in the constitutively tolerant cultivar *S. phureja*. In this plant, trehalose levels reached concentrations equal to those of other carbohydrates, giving evidence that the sugar acts as an osmoprotectant rather than merely playing a regulatory function as has been suggested in other situations [85]. Expression of carbohydrate-related genes during cold exposure matches these metabolite profiles in cultivar Desiree, showing the upregulation of sucrose synthase and galactinol synthase, leading to galactinol and eventually raffinose accumulation [77]. Free polyamine accumulation has been demonstrated to be involved in abiotic stress tolerance in other species [86, 87]. This was also detected in potatoes where free polyamine metabolism was affected by cold as shown through an upregulation of

arginine decarboxylase, *S*-adenosylmethionine decarboxylase, and spermidine synthase genes [77, 88]. Potato plants transformed with an apoplastic-localized yeast invertase had a greater invertase activity, higher content of sugar, and produced significantly less lipid peroxidation activity when exposed to low temperature (3 °C) and freezing temperatures (−1 or −9 °C) [89, 90]. The authors suggested that the improved cold tolerance was attributed to the stabilizing effect of sugar on the membranes.

Cell signaling is one of the most important cellular responses to cold treatment. Potatoes possess at least three calcium/calmodulin signaling proteins that display differential expression in response to cold stress in leaves after cold acclimation, and when combined with osmotic stresses [91]. Plant hormones, such as ABA pretreatment confers freezing tolerance to microplants transferred to soil, with no significant negative long-term effects on tuber production. Responses to ABA were found to be associated with increased antioxidant enzymatic activities of peroxidase and APX and decreased H_2O_2 content in the induction of freezing tolerance in the potato [92]. Antistress effects of salicylates can also be used in a planned manner to improve *in vitro* culture technology and hardening in potatoes for induction of tolerance to freezing in microplants after transplanting them to soil [93]. It appears that at least two mechanisms are involved in the induction of freezing tolerance in potato by salicylic acid (SA). One mechanism, exemplified in the cold-sensitive cultivar Atlantic, appears to involve induction of hydrogen peroxide (H_2O_2) accumulation leading to enhanced CAT activities, while another mechanism exemplified in the more cold-tolerant cultivar Alpha does not appear to involve H_2O_2 accumulation or enhanced CAT activities [94].

ROS generated during cold stress are involved in inducing the oxidative stress during chilling and in triggering cold-induced damage [95]. ROS can either act as signals that induce protection mechanisms or accelerate injury [96]. Freezing tolerance is attributed to the protective effect of sugars caused by their ability to scavenge ROS nonspecifically under stress conditions [97, 98].

44.5.2
Gene Functions and Omics

Protective proteins are produced under cold stress conditions. Pruvot *et al.* [99] identified CDSP34 protein that accumulates in the chloroplast in response to low temperatures. This protein plays a role in the structural mechanisms involved in the thylakoid tolerance to stress. A chaperonin protein Cpn60β involved in sustaining proper protein folding under stress was found to be constitutively expressed at a higher level in cold-tolerant potato species *S. commersonii*, but not in cold-susceptible *S. tuberosum* [100]. Dehydrins are believed to act as emulsifiers or chaperones in the cells by protecting proteins and membranes against unfavorable structural changes caused by dehydration. One of the dehydrin proteins, dhn2, was found to express at a higher level in cold-tolerant potato species [100]. Furthermore, the cold acclimation that improved the freezing tolerance in *S. commersonii* was associated with the accumulation of the transcripts of Scdhn2 [96]. A similar response to cold acclimation

was also observed for DHN24 (a SK3-type dehydrin protein) [101] and TAS14 [88]. As mentioned previously, overexpression of DHN4 in potatoes did not provide any benefit with respect to cold tolerance, although in the same trials, overexpression of mitochondrial MnSOD3:1 did confer tolerance [48].

In low-temperature stress trials conducted under controlled environment and in the field, lines overexpressing a heat-stable, LEA group 3-like protein from bromegrass (ROB5) had greater tolerance to freezing temperatures than the parental line [48]. Another class of proteins commonly studied in relation to cold and freezing tolerance are antifreeze proteins (AFPs) that inhibit ice growth and recrystallization. Expression of a synthetic AFP (similar to type 1 AFP of winter flounder) in cultivar Russet Burbank conferred freezing tolerance as assessed by electrolyte release analysis of the transgenic plant [102].

Many transcription factors regulate gene expression under cold conditions. Transgenics overexpressing several transcription factors (ERF, EREBP, DREB, and CBF) have improved freezing tolerance in potato plants. Ethylene-responsive factors (ERFs) are plant-specific transcription factors, many of which have been linked to plant defense responses. Overexpression of CaPF1, an ERF/AP2-type pepper transcription factor gene, effectively increased tolerance to freezing in potatoes [103]. In addition, StEREBP1 (ethylene-responsive element binding protein 1) is a transcription factor that responds to several environmental stresses. Overexpression of StEREBP1 enhanced tolerance to cold stress (growth at 8–10 °C for 2 months) in transgenic potato plants [104]. Overexpression of *Arabidopsis* rd29A::DREB1A also enhances freezing tolerance in transgenic potatoes [105]. Even in the absence of cold treatment, ectopic AtCBF overexpression improved the freezing tolerance of transgenic *Solanum* species [106]. In the meantime, overexpression of ectopic AtCBF1 affected many alterations associated with cold acclimation such as thickening of leaves and increase in proline and total sugar contents. The leaves of these transgenic *S. commersonii* were darker green, had higher chlorophyll and lower anthocyanin levels, had greater stomatal numbers, and displayed greater photosynthetic capacity, suggesting their higher productivity potential [107].

It is worth mentioning several other genes. For instance, potato 1-aminocyclopropane-1-carboxylate oxidase genes, ACO1 and ACO2, can be induced by cold stress (0 °C), but are differentially expressed by other stresses such as heat, wounding, and soil flooding [108]. Renaut [109] presented a series of studies on the cold response in two genotypes of *S. tuberosum*, PS3 and Desiree. Oufir *et al.* [77] focused on carbohydrates and polyamines because they are known cryoprotectants associated with cold acclimation. They used transcriptomics and metabolomics approaches to demonstrate how three genotypes of potato responded to chilling exposure: a tetraploid *S. tuberosum* (cultivar Desiree) was not able to acclimate to cold, a dihaploid *S. tuberosum* (PS3) acclimated to cold, and *S. phureja* (CHS) was more tolerant to cold on a constitutive level. Although free polyamine accumulation was not pronounced upon cold exposure, an array of genes involved in several other metabolisms, for example, amino acid, carbohydrate, energy, detoxification, and photosynthesis, were differentially expressed in these potatoes under cold exposure.

44.5.3
Cold-Induced Sweetening during Storage

Refrigeration is the most important and effective technology employed to maintain the postharvest quality of potatoes. Storage at cold temperatures prevents sprouting, minimizes disease losses, reduces shrinkage, and improves the retention of dry matter and extends the marketability, thus supplying consumers and the processing industry with high-quality tubers throughout the year. Unfortunately, when tubers are stored at temperatures between 2 and 4 °C, they undergo a phenomenon known as cold sweetening or low-temperature sweetening (LTS). At these temperatures, the rate of conversion of starch to reducing sugars (i.e., glucose and fructose) is accelerated. These potatoes are unacceptable for processing into chips or French fries because when cooked in oil at high temperatures, the accumulated reducing sugars react with free amino acids in the potato cells, forming a brown to black pigmented and bitter-tasting product via a nonenzymatic, Maillard-type reaction [110, 111]. Such products also have elevated amounts of acrylamide, a neurotoxin and potential carcinogen [111]. Therefore, LTS is a major concern since all commercial potato cultivars used for the production of potato chips and fries accumulate excess free reducing sugars when exposed to cold stress.

The mechanism of LTS is somewhat understood. Low-temperature storage of potato tubers induces amylolytic enzymes that initiate the breakdown of starch stored in the amyloplasts. The breakdown products, both hexose phosphates (hexose-P) and free sugars, are exported from the amyloplast to the cytosol where they are converted to sucrose [112]. Sucrose-phosphate synthase produces sucrose 6-phosphate (Suc6P) and UDP from UDP-glucose and D-fructose 6-phosphate. Then, Suc6P is hydrolyzed by sucrose phosphatase (SPP) to yield sucrose and inorganic phosphate (Pi) [113]. This sucrose can then be hydrolyzed into its constituent hexoses by soluble acid invertase [114].

A genomic investigation by the Canadian Potato Genome Project initiative (www.cpgp.ca) [115] sheds some light on LTS. In this study, mature, harvested tubers of cultivar Shepody were stored at 4 °C for 3 months. Normalized library was constructed and ESTs were sequenced. Among the 5000 ESTs identified, only 7 transmembrane proteins Mlo8 and putative protein transport protein SEC13 were highly regarded, while others were marked unknown. With the complete genome sequencing data like to soon become available, these genes could be revisited for their functions related to LTS.

At present, sucrose phosphatase and the vacuolar acid invertase (VInv) are targeted in order to control LTS. Chen *et al.* [116] suppressed sucrose synthesis by RNAi-mediated silencing of SPP expression, leading to an accumulation of Suc6P. Overall, SPP-silenced tubers exhibited only minor differences in total soluble carbohydrate accumulation. However, the sucrose to hexose conversion was greatly reduced because of an unexpected blocking of cold-induced expression of VInv. Bhaskar *et al.* [111] used a targeted RNAi approach to demonstrate that the potato VInv is responsible for reducing sugar accumulation in cold-stored tubers. Even when tubers

were stored at 4 °C, potato chips processed from VInv-silencing lines were light in color and showed a 15-fold reduction in acrylamide [111]. In a more traditional breeding approach, Hamernik *et al.* [117] achieved a similar goal by introgressing wild species germplasm with extreme resistance to cold sweetening at very low storage temperatures (2 °C) into the cultivated potato. Selected accessions were crossed as males to haploids of *S. tuberosum* to produce adapted hybrids, which produced good tuber type and low levels of reducing sugars under extremely low storage temperatures [117]. Interestingly, comparable low levels of VInv gene expression were observed in cold-stored tubers of VInv silencing lines and wild potato germplasm stocks that are resistant to cold-induced sweetening. These results demonstrate that both processing quality and acrylamide problems in potato can be controlled effectively by suppression of the VInv gene through biotechnology or targeted breeding.

Several other attempts had been made to control LTS, by manipulating VInv activity through the ectopic expression of an invertase inhibitor [118–120], through antisense inhibition [114], or through RNAi suppression [121, 122]. The success of these efforts was limited because invertase activity was only partially reduced. It seems a nearly complete silencing of the VInv gene is required to effectively control LTS [111].

44.6 Salt Tolerance

Soil salinity is defined as excess sodium chloride (NaCl) in soil. Plants encounter salt stress when grown in naturally occurring saline soils or when irrigated with saline water. According to the review by Donnelly *et al.* [123], most of the areas in the world under potato cultivation are in countries that are not overly affected by salinity. The exceptions, as pointed out by the authors, are countries in Southern and Southeastern Asia. Since China and India have become the top two potato producers in the world (producing over 30% of the world potatoes), the salinity problem could become a major issue for these two countries. In order to maintain production levels in these countries, potato cultivars with improved stress tolerance to heat, drought, and salinity are the top priority.

Irrigation has become an important agricultural practice, which is employed to produce up to 30% of the world's food using 15% of the cultivated land area. Salt injury occurs when high-concentration salt water comes in contact with above- or underground plant parts. This contact will lead to withdrawal of water from the plant tissues due to osmotic pressure. The injured tissue cannot resume normal functions and lead to necrosis.

Mechanisms of salinity tolerance in plants are related to a combination of plant stresses, including drought stress, ion toxicity, mineral deficiency, and oxidative stress. Because salinity affects major biochemical processes of the cell, such as protein synthesis, energy generation, photosynthesis, and lipid metabolism, plants use diversified mechanisms to counter these effects. These include limiting the

uptake and transport of selected ions, compartmentalization in cells and organs, altered cellular and organelle membrane structures, producing antioxidant compounds and enzymes, and using alternative biosynthetic pathways. Early studies showed that proline, a compatible solute as mentioned earlier, is accumulated in the saline-stressed potatoes. Potatoes were considered as moderately salt-sensitive in early studies. However, significant variations in salt tolerance among *S. tuberosum* cultivars are expected, but they have not been explored systematically. Some field, greenhouse, and *in vitro* evaluations have been completed as summarized in Donnelly *et al.* [123]. As already mentioned, plants' response to salt is also related to the responses to other stresses, as demonstrated by microarray analysis of leaf responses to cold and salt stresses [76].

44.6.1 Mechanisms of Salt Response

Major changes in potato leaves in response to salt exposure were found to be the repression of photosystems I and II and chlorophyll synthesis, according to microarray analyses [124]. This was mirrored by protein data in which the most drastically downregulated proteins were involved in photosynthesis and protein synthesis [125]. In addition, changes in gene expression of carbohydrate and amino acid metabolism suggested that salt stress caused modifications at the metabolic level.

Studies have identified cell signaling proteins from potatoes that are induced by salt stress, including a novel leucine-rich repeat receptor-like kinase, StLRPK1 [126], and calcium/calmodulin signaling components ScCaM1 and ScCaM5 [91]. Catecholamines are synthesized in response to both ABA and salt stress, and are proposed to be stress agent compounds that play an important role in the regulation of starch–sucrose conversion in plants and may be implicated in several other functions including wounding and pathogen responses [127]. Expression of StPUB17, an UND/PUB/ARM repeat E3 ubiquitin ligase, is upregulated by many abiotic stresses including salt, and StPUB17-silenced plants were more susceptible to both salt stress and *Phytophthora infestans* [128]. Nitric oxide appears to interact with salt stress signaling according to studies on *Arabidopsis*. *Arabidopsis* mutants of the AtnOA1 gene involved in nitric oxide synthesis are more sensitive to salt stress, but transgenic expression of StNOA1 from *S. tuberosum* was able to return salt sensitivity to wild-type level [129–132].

In response to salinity, cells accumulate osmoprotectants, small molecules that balance the osmotic difference between the cell's surroundings and the cytosol. Proline is a small molecule that accumulates upon salt exposure and is believed to protect cells via a mechanism similar to that proposed for drought tolerance. *N*-succynilarginine is induced under salt stress and it is involved in arginine metabolism that leads to the production of ornithine and proline [125]. Overexpression of Δ1-pyrroline-5-carboxylate synthetase increases proline production and confers salt tolerance in transgenic potato plants [133]. Lycine-betaine (GB) is a common compatible solute that accumulates in many higher plant species in response to salinity, drought, and low temperature [134], but potato is betaine deficient. It has been demonstrated in a variety of studies that GB exerts protective

effects and stabilizes macromolecules, enzyme activities, and membranes under stressful conditions [135, 136]. Transgenic plants with enhanced salt tolerance were engineered with the ability to synthesize GB in chloroplasts via the introduction of the bacterial choline oxidase (codA) gene under the control of an oxidative stress-inducible SWPA2 promoter [137]. Ning *et al.* [138] also created transgenic potato plants expressing a gene for GB synthesis that had improved tolerances to drought and salinity. Pruvot *et al.* [99] identified two proteins that accumulate in response to salt: CDSP 32 is suggested to be involved in osmoregulation in the stroma and CDSP 34 is postulated to play a role in the structural mechanisms involved in the tolerance of thylakoids to dehydration stress [99].

A cDNA microarray analysis of leaves reveals that salt stress activated several HSPs, late-embryogenesis abundant proteins, and dehydrins [124]. The accumulation of HSPs can act as chaperone to facilitate the correct folding of proteins and protect them from denaturing under stress conditions. The leaves of potatoes exposed to salt stress induced the gene expression of several HSPs [124] and salt-tolerant cultivars have a propensity to accumulate more HSPs under salt stress [125]. Cpn60b is another example of a molecular chaperone from *Solanum* that is induced by salt stress [100]. These authors first identified this gene along with several others in a functional screen for salt tolerance using a heterologous expression method in *E. coli*. Another of these candidate salt stress-related genes was dhn2, a dehydrin that shares homology with the D11 group of LEA dehydrins and is synthesized during seed desiccation and in response to salt stress.

Several hormones have been implicated in responses to salt stress. For example, calreticulin is a Ca^{2+} storage protein that appears to be involved in ABA-induced salt tolerance. Its gene expression and protein levels are induced by salt stress [139]. Furthermore, grafting experiments demonstrate that both salt stress tolerance and calreticulin expression are regulated by the roots [139]. Experiments with acetylsalicylic acid suggest that pretreatment induces preadaptive responses to salt and water stresses leading to the protection of the photosynthetic pigments and the maintenance of membrane integrity, which is ultimately reflected in improved plant growth [140].

Salt transporters/vacuolar Na^+/H^+ antiporters have been studied for salt response. Plant cells adapting to salt stress improve cellular ion homeostasis by accumulating organic solutes in the cytosol, by compartmentalizing ions in the vacuole, and by excluding extra Na^+ ions from the cells. To this end, transgenic potato plants constitutively overexpressing an *Arabidopsis* tonoplast Na^+/H^+ antiporter (AtNHX1 gene) were constructed but not analyzed for salt tolerance [141]. Another group by Bayat *et al.* [142] transformed two cultivars of potatoes (*S. tuberosum*) with a barley antiporter gene (HvNHX2) driven by the CaMV 35S promoter. Transgene expression conferred a higher NaCl tolerance to one of the cultivars.

44.6.2
Genes Related to Salt Tolerance

Altering potato metabolism through transgenic approaches has also altered salt tolerance. Besides the genes indicated above, several others have been studied.

Transgenic plants with reduced StubGAL83 expression had increased sensitivity to salt stress, as well as impaired root and tuber development [143]. This potato gene was suspected to be an important regulator of salt stress because it encodes a subunit of a protein kinase complex that is similar to the yeast SNF1 and mammalian AMPK complexes that are modulated by changes in the cellular AMP/ATP ratio and are important regulators of metabolic and stress responses. Conversely, continuous expression of a glyceraldehyde-3-phosphate dehydrogenase in transgenic potato plants resulted in improved tolerance against salt loading [144]. The importance of nitrogen status in metabolism has been highlighted in salt-stressed potato plants that had increased glutamine synthetase activity in the roots and decreased activity in the leaves [145].

Salt stress response is also related to biotic stress factors. Microarray analysis of leaves reveals that salt stress induced the expression of several pathogenesis-related proteins, as well as several transcription factors related to plant defense pathways, demonstrating a crosstalk between abiotic and biotic stress responses during salt exposure [124]. In another study, 6 of 20 proteins upregulated by salt stress were known to play a role in plant defense (i.e., osmotin-like protein, HSPs, calreticulin, and protease inhibitors) highlighting the close link between these processes [125]. For example, osmotin is a member of the pathogenesis-related family of proteins 5 (PR-5), which is induced by biotic stresses and implicated in defense against fungi [146, 147]. However, osmotin protein levels in salt-tolerant cultivars are also upregulated in response to salt. Furthermore, overexpression of osmotin has been proposed to confer salt tolerance to transgenic potato plants [148]. PR-10a is another pathogenesis-related protein with increased protein expression in potato cell cultures under salt stress. Potato cell cultures that overexpressed a PR-10a transgene were conferred increased salt and osmotic tolerance [149].

Transcription factors are involved in osmotic stress response via ABA-dependent or ABA-independent pathways. Functional studies of several transcription factors have demonstrated their effectiveness in mitigating salt stress [124]. For example, *Arabidopsis*DREB/CBF (dehydration-responsive element binding/C-repeat binding factor) proteins are key transactivational factors involved in environmental stresses such as cold, drought, and salinity [105]. Improved tolerance to salinity was conferred to potato plants transformed with *Arabidopsis* DREB1A under the control of an *Arabidopsis* stress-inducible promoter (rd29A) [150]. Ectopic expression of potato StZFP1 (a TFIIIA-type zinc finger protein), also driven by rd29A, in transgenic tobacco increased plant tolerance to salt stress [53]. StEREBP1 (ethylene-responsive element binding protein 1) is a transcription factor that binds GCC and DRE/CRT *cis*-elements and that responds to several environmental stresses including low temperature. Overexpression of StEREBP1 in potatoes enhanced their tolerance to cold and salt stress [104].

44.6.3 Omics Studies

Salinity tolerance is a multigenic trait with complex regulatory factors. Significant progress in its understanding has been made in *Arabidopsis* and tomato; however,

breeding for salinity tolerance in potato is still in its infancy. Several tolerant lines of cultivated and wild species have been identified on the basis of the Center for Genetic Resources (CGN) Genebank evaluation data, but breeding a tolerant line with growth vigor and yield is still a long process. To aid in this endeavor, several transcriptome-wide analyses have been performed in order to identify potato genes involved in salinity. As previously mentioned, Rensink *et al.* [75, 76] profiled genes in response to salt and temperature stresses. Other omics studies include a microarray study by Legay *et al.* [124] and a proteomics study by Aghaei *et al.* [125]. Both described the expression of several individual genes or proteins and several functional categories of genes that are differentially regulated under salinity.

Salt tolerance of potato cultivars and clones in general has increased activity of antioxidant enzymes, including SOD and peroxidase (POD) [151–153] as well as APX, CAT, and glutathione reductase (GR) [154]. Similarly, treatments that boost the antioxidant capacity of a plant, such as exogenous application of ascorbic acid, have also been found to ameliorate the salinity tolerance and increase the CAT and SOD activities in *S. tuberosum* [155].

Several examples of successful transgenic approaches have manipulated the antioxidant system to improve salt tolerance. For example, transgenic *in vitro* plants expressing a bacterial CAT gene had an improved multiplication rate under salt stress compared to control, while knockdown of the CAT gene reduced the multiplication rate, tuber yield, and leaf chlorophyll content [156]. Another study expressed *Arabidopsis* nucleoside diphosphate kinase 2, a known regulator of antioxidant gene expression, under the control of anoxidative stress-inducible promoter (SWPA2). The transgenic potato plants had higher APX activity and were more tolerant to high salt concentrations, presumably because of improved scavenging of ROS derived from salt stress [56]. This same research group demonstrated that transgenic potato plants expressing Cu/Zn SOD and APX genes in chloroplasts under the control of the SWPA2 promoter had increased tolerance to salt stress. Furthermore, retransformation of these same transgenic plants with a bacterial choline oxidase (coda) gene to synthesize glycine-betaine in chloroplast synergistically enhanced salt tolerance. Ectopic production of glycine-betaine in the chloroplast helped to maintain higher activities of SOD, APX, and CAT during salt stress [58]. A similar transgenic approach engineered potato plants with enhanced ascorbic acid accumulation and tolerance to salt stress by overexpression of a rat GLOase gene that is responsible for ascorbic acid production [157]. The T1 transgenic plants exposed to salt stress (100 mM NaCl) survived better with increased shoot and root length compared to untransformed plants. The elevated level of AsA accumulation in transgenics was directly correlated with their ability to withstand abiotic stresses.

44.6.4 Wounding

Tuber skin is a suberized layer of native periderm. It is often called skin-set. Tuber skin is used to protect tubers from pathogen infection, desiccation, and water loss. Tuber skins can be easily wounded by mishandling during harvest and postharvest

storage, resulting in decreased tuber quality and increased defects. The cultivars that lack an effective suberization function will be more prone to tuber defects such as bruising, crack, and skinning. In some cases, within 1 h of wounding, tuber skin tissues respond with wound-induced suberization [158]. The overall wound-healing process in potato tubers is characterized by the rapid accumulation of waxes to restrict water vapor loss [159] and the development of a suberized closing layer and associated wound periderm that resists desiccation and microbial invasion [160]. Suberization requires the biosynthesis of phenolic, aliphatic, and glycerol monomers and assembly of these monomers into polymer domains, named as suberin poly (phenolic) domain (SPPD) and suberin poly (aliphatic) domain (SPAD) [161]. Phenylalanine ammonia lyase (PAL) is a major player in the regulation of wound-healing activity because it is required for the formation of polyphenols in the SPPD [160]. In relation to this, a proteomic analysis of the wound-healing process reveals the accumulation of several peroxidases [162] that have been postulated to participate in the cross-linking of the hydroxycinamic alcohols that constitute lignin and SPPD [163].

Three hormones have been traditionally associated with wound healing because their levels dramatically increased upon wounding [164]. Most recent evidence implicates ABA in wound healing [164], but does not support a role for ethylene in this process, while the role of jasmonic acid remains speculative [164]. This is further supported by the observation that wound-healing ability declines with tuber age/storage, partly because of a reduced ability to accumulate ABA that appears to modulate PAL activity and accumulation of suberin polyphenols [160].

Chaves *et al.* [162] provide insight into the proteomics behind the wound-healing process in tuber slices. The cell differentiation processes that were triggered by slicing lead to changes in metabolism, activation of defense, and cell wall reinforcement. Proteins detected related to storage, for example, patatin, cell growth and division, cell structure, signal transduction, energy production, disease/defense mechanisms, secondary metabolism, and suberization. Even 8 days after wounding, the protein patterns of slices of wound periderm and native periderm were still quite different [162].

To further understand the metabolites associated with suberization process, a GC/MS-based metabolite profiling study was conducted, using wound-healing potato (*S. tuberosum* L.) tubers [165]. Using principal component analysis methods, the authors revealed a separation of metabolite profiles according to different suberization stages, with clear temporal differences in the nonpolar and polar profiles. These temporal differences were in keeping with earlier histochemical analyses of suberin macromolecular assembly: first, the phenolic compounds that accumulate in response to a wounding event are polymerized into the SPPD within the primary cell wall; then, this event is closely followed by the biosynthesis of SPAD components and their assembly into a multilammelar structure between the cell wall and the plasma membrane [161, 166]. Yang *et al.* [165] observed that the nonpolar metabolite profiles contained characteristic SPAD components, which appeared later than the bulk of the SPPD components apparent in the polar metabolite profiles. In the nonpolar profiles, suberin-associated aliphatics contributed the most to cluster formation, while a broader range of metabolites (including organic acids, sugars, amino acids, and phenylpropanoids) influenced cluster formation among polar profiles. The authors exploited strong

correlations between known suberin-associated compounds and several unidentified metabolites in the profiles to identify novel compounds involved in suberin biosynthesis. In addition, the results distinguished between suberin-related metabolites and metabolites associated with other wound-induced processes. For example, chlorogenic acid was clearly identified as one of the phenoic compounds induced by wounding.

In addition to suberization, wounding of potato tubers induces changes in some other metabolites, including those that function as defense compounds, for example, hydroxycinnamoyl putrescines [165, 167] and chlorogenic acid [165, 168]. Plants appear to have distinct signal transduction pathways that can distinguish between insect damage and abiotic damage based on the presence of insect-derived elicitors that function to induce plant defense against herbivory [169, 170]. For example, Turra *et al.* [171] have demonstrated differential expression patterns of potato protease inhibitors in response to wounding and nematode infection. Nevertheless, during the wound-healing process, a number of upregulated proteins result in the production of antimicrobial compounds such as phenols and pathogenesis-related (PR) peptides or the protein themselves are PR, such as beta-1,3-glucanase (PR-2), chitinases (PR-3), osmotins (PR-5), protease inhibitors (PR-6), plant peroxidases (PR-9), and PR-10 proteins [162]. The induction of multiple protease inhibitors upon wounding [162] is of particular interest because after patatin, low molecular weight proteinase inhibitors are the most abundant group of tuber storage proteins [172] and they have been implicated in the regulation of endogenous protease activity, protein stabilization, modulation of apoptosis, and protection from biotic stress [173, 174].

In addition to its function in defense [165, 168], chlorogenic acid is a major chemical involved in a nonenzymatic discoloration in potato tubers, called after-cooking darkening (ACD) [175]. This leads to another study that recently completed. Murphy *et al.* [176] used a comparative proteomics approach to identify proteins related to potato tuber ACD, a defect not welcomed by French fry industry. Clustering analysis of relative quantitative proteomics data revealed a cluster of proteins whose relative expression appeared the most positively correlated with darkening and an additional, smaller cluster of proteins, negatively correlated with darkening. Perhaps, most interestingly, they observed multiple proteins related to lipid signaling and protease inhibitor-based wound responses that are correlated with tissue darkening. The changes in relative protein abundance showed an enhanced wound response program in high ACD tissues. Among the wound-induced proteins, five were suggested by the authors for further investigation. They are polyphenol oxidase, aspartic protease inhibitor 7 precursor, 5-lipoxygenase, linoleate:oxygen oxidoreductase, and patatin T5 precursor. The authors suggest this wound response occurs in parallel to an increase in polyphenol synthesis, leading to tissue darkening [176].

44.7
Conclusions and Future Perspectives

Environmental stresses, both during growing season and during postharvest storage, can affect the marketable quality of the potato crop. Stresses during the growing

season reduce photosynthetic efficiency and function, resulting in varied metabolism changes and inhibition of the optimal growth and development of tubers. Recent efforts in genomics, transcriptomics, proteomics, and metabolomics to understand the physiology, biochemistry, and molecular genetics of stress responses have provided insight into these processes. Many promising genes have been identified and their roles and effectiveness in stress tolerance will be tested. Unfortunately, one last hurdle lies in the fact that traditional transgenic approaches have not been welcomed by consumers and agricultural policy makers of the developed world. This resistance has inhibited the introduction of many stress-tolerant transgenic cultivars into world markets thus far. Nevertheless, recent advancement in intragenic approaches, as outlined by Rommens *et al.* [177, 178], show promise in developing new cultivars while reducing concerns about the use of selection markers and genes from foreign species. It is very likely that some well-defined new generation intragenic potato cultivars will be successfully tested under field conditions.

The completion of the potato genome sequencing in the near future will boost the study of abiotic stress significantly. Many genes and markers will be identified and developed using the existing breeding lines. Because of the complexity and the heterozygosity of the cultivated potato cultivars, association mapping method will become a more effective tool to identify genes and markers with much less time. Finally, marker-assisted selection could be more successful if omics data could be integrated into breeding processes for new cultivar development.

References

1 Bryan, G.J. and Hein, I. (2008) Genomic resources and tools for gene function analysis in potato. *Int. J. Plant. Genomics*, **2008**, 216513.

2 Li, X., Griffiths, R., De Koeyer, D., Rothwell, C., Gustafson, V., Regan, S. *et al.* (2008) Functional genomic resources for potato. *Can. J. Plant Sci.*, **88** (4), 573–581.

3 Duguay, J.L., XiuQing, L., and Regan, S. (2007) The potential for genomics in potato improvement. CAB reviews: perspectives in agriculture, veterinary science. *Nutr. Nat. Resour.*, **2** (075), 13–13.

4 FAO (2008) Potato World. Available at http://www.potato2008.org/en/world/index.html Accessed October 28, 2010.

5 FAO (2003) *Unlocking The Water Potential Of Agriculture*, FAO. Available at http://www.fao.org/DOCREP/006/Y4525E/Y4525E00.HTM. Accessed October 28, 2010.

6 Vos, J. and Haverkort, A.J. (2007) Water availability and potato crop performance, in *Potato Biology and Biotechnology* (eds D. Vreugdenhil, J. Bradshaw, C. Gebhardt, F. Govers, D.K.L. Mackerron, M.A. Taylor *et al.*), Elsevier Science BV., Amsterdam, p. 333.

7 Weisz, R., Kaminski, J., and Smilowitz, Z. (1994) Water deficit effects on potato leaf growth and transpiration: utilizing fraction extractable soil water for comparison with other crops. *Am. Potato J.*, **71** (12), 829–840.

8 Jefferies, R.A. and MacKerron, D.K.L. (1993) Responses of potato genotypes to drought. II. Leaf area index, growth and yield. *Ann. Appl. Biol.*, **122**, 105–112.

9 Levy, D. (1986) Genotypic variation in the response of potatoes (*Solanum tuberosum* L.) to high ambient temperatures and water deficit. *Field Crops Res.*, **15** (1), 85–96.

10 Dalla Costa, L., Delle Vedove, G., Gianquinto, G., Giovanardi, R., and Peressotti, A. (1997) Yield, water use efficiency and nitrogen uptake in potato: influence of drought stress. *Potato Res.*, **40** (1), 19–34.

11 Kumar, D. and Minhas, J.S. (1999) Effect of water stress on photosynthesis, productivity and water status in potato. *J. Indian Potato. Assoc.*, **26**, 7–10.

12 Martin, M.W. and Miller, D.E. (1987) Alterations in irrigation schedules and rates to eliminate potato genotypes susceptible to water stress. Abstracts of Conference Papers and Posters of the 10th Triennial Conference of the European Association for Potato Research, p. 21.

13 van Loon, C.D. (1981) The effect of water stress on potato growth, development, and yield. *Am. Potato J.*, **58** (1), 51–69.

14 Lynch, D.R. and Tai, G.C.C. (1989) Yield and yield component response of eight potato genotypes to water stress. *Crop Sci.*, **29**, 1207–1211.

15 Haverkort, A.J., Fasan, T., and van de Waart, M. (1991) The influence of cyst nematodes and drought on potato growth. 2. Effects on plant water relations under semi-controlled conditions. *Eur. J. Plant Pathol.*, **97** (3), 162–170.

16 Evans, K., Franco, J., and Descurrah, M.M. (1975) Distribution of species of potato cyst nematode in South America. *Nematologica*, **21** (365), 369.

17 Lahlou, O. and Ledent, J. (2005) Root mass and depth, stolons and roots formed on stolons in four cultivars of potato under water stress. *Eur. J. Agron.*, **22** (2), 159–173.

18 Haverkort, A.J., Van De Waart, M., and Bodlaender, K.B.A. (1990) The effect of early drought stress on numbers of tubers and stolons of potato in controlled and field conditions. *Potato Res.*, **33** (1), 89–96.

19 Martin, R.J., Jamieson, P.D., Wilson, D.R., and Francis, G.S. (1992) Effects of soil moisture deficits on the yield and quality of 'Russet Burbank' potatoes. *N. Z. J. Crop Hortic. Sci.*, **20**, 1–9.

20 Valliyodan, B. and Nguyen, H.T. (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Curr. Opin. Plant. Biol.*, **9** (2), 189–195.

21 Liu, F., Jensen, C.R., Shahanzari, A., Andersen, M.N., and Jacobsen, S. (2005) ABA regulated stomatal control and photosynthetic water use efficiency of potato (*Solanum tuberosum* L.) during progressive soil drying. *Plant. Sci.*, **168** (3), 831–836.

22 Wu, G., Shao, H., Chu, L., and Cai, J. (2007) Insights into molecular mechanisms of mutual effect between plants and the environment: a review. *Agron. Sustain. Dev.*, **27** (1), 69–78.

23 Mane, S.P., Robinet, C.V., Ulanov, A., Schafleitner, R., Tincopa, L., Gaudin, A. *et al.* (2008) Molecular and physiological adaptation to prolonged drought stress in the leaves of two Andean potato genotypes. *Funct. Plant Biol.*, **35** (8), 669–688.

24 Schafleitner, R., Rosales, R.O.G., Gaudin, A., Aliaga, C.A.A., Martinez, G.N., Marca, L.R.T. *et al.* (2007) Capturing candidate drought tolerance traits in two native Andean potato clones by transcription profiling of field grown plants under water stress. *Plant Physiol. Biochem.*, **45** (9), 673.

25 Vasquez-Robinet, C., Mane, S.P., Ulanov, A.V., Watkinson, J.I., Stromberg, V.K., De Koeyer, D. *et al.* (2008) Physiological and molecular adaptations to drought in Andean potato genotypes. *J. Exp. Bot.*, **59** (8), 2109–2123.

26 Watkinson, J.I., Hendricks, L., Sioson, A.A., Vasquez-Robinet, C., Stromberg, V., Heath, L.S. *et al.* (2006) Accessions of *Solanum tuberosum* ssp. andigena show differences in photosynthetic recovery after drought stress as reflected in gene expression profiles. *Plant Sci.*, **171** (6), 745–758.

27 Schafleitner, R. (2009) Growing more potatoes with less water. *Trop. Plant Biol.*, **2** (3), 111–121.

28 Min, F., LiPing, J., SanWen, H., KaiYun, X., QingChang, L., and DongYu, Q. (2008) Effects of drought on gene expressions of key enzymes in carotenoid and flavonoid biosynthesis in potato. *Acta Hortic. Sin.*, **35** (4), 535–542.

29 Morgan, J.M. (1984) Osmoregulation and water stress in higher plants. *Annu. Rev. Plant Physiol.*, **35** (1), 299–319.

30 Kavi Kishor, P.B., Sangam, S., Amrutha, R.N., Sri Laxmi, P., Naidu, K.R., Rai, K.R.S.S. *et al.* (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Curr. Sci.*, **88** (3), 15.

31 Wood, A.J. (1999) Comparison of salt-induced osmotic adjustment and trigonelline accumulation in two soybean cultivars. *Biol. Plant.*, **42** (3), 389–394.

32 Cho, Y., Njiti, V.N., Chen, X., Lightfoot, D.A., and Wood, A.J. (2003) Trigonelline concentration in field-grown soybean in response to irrigation. *Biol. Plant.*, **46** (3), 405–410.

33 De Ronde, J.A., Cress, W.A., Krüger, G.H.J., Strasser, R.J., and Van Staden, J. (2004) Photosynthetic response of transgenic soybean plants, containing an *Arabidopsis* P5CR gene, during heat and drought stress. *J. Plant Physiol.*, **161** (11), 1211–1224.

34 Simon-Sarkadi, L., Kocsy, G., Varhegyi, A., Galiba, G., and de Ronde, J.A. (2005) Genetic manipulation of proline accumulation influences the concentrations of other amino acids in soybean subjected to simultaneous drought and heat stress. *J. Agric. Food Chem.*, **53** (19), 7512–7517.

35 Evers, D., Lefvre, I., Legay, S., Lamoureux, D., Hausman, J., Rosales, R.O.G. *et al.* (2010) Identification of drought-responsive compounds in potato through a combined transcriptomic and targeted metabolite approach. *J. Exp. Bot.*, **61** (9), 2327–2343.

36 Weiser, R.L., Wallner, S.J., and Waddell, J.W. (1990) Cell wall and extensin mRNA changes during cold acclimation of pea seedlings. *Plant Physiol.*, **93** (3), 1021–1026.

37 Ueda, A., Yamamoto-Yamane, Y., and Takabe, T. (2007) Salt stress enhances proline utilization in the apical region of barley roots. *Biochem. Biophys. Res. Commun.*, **355** (1), 61.

38 Sobeih, W.Y., Dodd, I.C., Bacon, M.A., Grierson, D., and Davies, W.J. (2004) Long-distance signals regulating stomatal conductance and leaf growth in tomato (*Lycopersicon esculentum*) plants subjected to partial root-zone drying. *J. Exp. Bot.*, **55** (407), 2353–2363.

39 Schafleitner, R., Gutierrez, R., Espino, R., Gaudin, A., Pérez, J., Martínez, M. *et al.* (2007) Field screening for variation of drought tolerance in *Solanum tuberosum* L. by agronomical, physiological and genetic analysis. *Potato Res.*, **50** (1), 71–85.

40 Watkinson, J.I., Hendricks, L., Sioson, A.A., Heath, L.S., Bohnert, H.J., and Grene, R. (2008) Tuber development phenotypes in adapted and acclimated, drought-stressed *Solanum tuberosum* ssp. *andigena* have distinct expression profiles of genes associated with carbon metabolism. *Plant Physiol. Biochem.*, **46** (1), 34–45.

41 Merlot, S., Gosti, F., Guerrier, D., Vavasseur, A., and Giraudat, J. (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant J.*, **25** (3), 295–303.

42 Meinhard, M., Rodriguez, P.L., and Grill, E. (2002) The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signalling. *Planta*, **214** (5), 775–782.

43 Chen, Y.M., Ferrar, T.S., Lohmeier-Vogel, E.M., Morrice, N., Mizuno, Y., Berenger, B. *et al.* (2006) The PII signal transduction protein of *Arabidopsis thaliana* forms an arginine-regulated complex with plastid *N*-acetyl glutamate kinase. *J. Biol. Chem.*, **281** (9), 5726–5733.

44 Konrad, Z. and Bar-Zvi, D. (2008) Synergism between the chaperone-like activity of the stress regulated ASR1 protein and the osmolyte glycine-betaine. *Planta*, **227** (6), 1213–1219.

45 Chaves, M.M., Maroco, J.P., and Pereira, J.S. (2011) Understanding plant responses to drought: from genes to the whole plant. *Funct. Plant Biol.*, **30**, 239–264.

46 Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M. *et al.* (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in

stress tolerance in *Arabidopsis thaliana*. *Plant J.*, **29** (4), 417–426.

47 Liu, H., Dai, X., Xu, Y., and Chong, K. (2007) Over-expression of OsUGE-1 altered raffinose level and tolerance to abiotic stress but not morphology in *Arabidopsis*. *J. Plant Physiol.*, **164** (10), 1384.

48 Waterer, D., Benning, N.T., GuoHai, W., XiMing, L., XunJia, L., Gusta, M. *et al.* (2010) Evaluation of abiotic stress tolerance of genetically modified potatoes (*Solanum tuberosum* cv. Desiree). *Mol. Breed.*, **25** (3), 527–540.

49 Coleman, W.K. (2008) Evaluation of wild *Solanum* species for drought resistance. 1. *Solanum gandarillasii* Cardenas. *Environ. Exp. Bot.*, **62** (3), 221–230.

50 Colmenero-Flores, J.M., Campos, F., Garciarrubio, A., and Covarrubias, A.A. (1997) Characterization of *Phaseolus vulgaris* cDNA clones responsive to water deficit: identification of a novel late embryogenesis abundant-like protein. *Plant Mol. Biol.*, **35** (4), 393–405.

51 Sun, W., Bernard, C., Van Cotte, B.D., Van Montagu, M., and Verbruggen, N. (2001) At-HSP17.6A, encoding a small heat-shock protein in *Arabidopsis*, can enhance osmotolerance upon overexpression. *Plant J.*, **27** (5), 407–415.

52 Fan, M., Jin, L.P., and Huang, S.W. (2007) Cloning and expression of a full-length cDNA of SoFtsH gene in potato under drought stress. *Acta Agron. Sin.*, **33**, 1748–1754.

53 Tian, Z.D., Zhang, Y., Liu, J., and Xie, C.H. (2010) Novel potato C2H2-type zinc finger protein gene, StZFP1, which responds to biotic and abiotic stress, plays a role in salt tolerance. *Plant. Biol. (Stuttg.)*, **12** (5), 689–697.

54 Stiller, I., Dulai, S., Kondrák, M., Tarnai, R., Szabó, L., Toldi, O. *et al.* (2008) Effects of drought on water content and photosynthetic parameters in potato plants expressing the trehalose-6-phosphate synthase gene of *Saccharomyces cerevisiae*. *Planta*, **227** (2), 299–308.

55 Van Der Mescht, A., De Ronde, J.A., Slabbert, M.M., and Oelofse, D. (2007) Enhanced drought tolerance in transgenic potato expressing the *Arabidopsis thaliana* Cu/Zn superoxide dismutase gene. *S. Afr. J. Sci.*, **103** (3–4), 169–173.

56 Tang, L., Kim, M.D., Yang, K., Kwon, S., Kim, S., Kim, J. *et al.* (2008) Enhanced tolerance of transgenic potato plants overexpressing nucleoside diphosphate kinase 2 against multiple environmental stresses. *Transgenic Res.*, **17** (4), 705–715.

57 Tang, L., Tang, H., and Kwak, S.S. (2008) Improving potato plants oxidative stress and salt tolerance by gene transfer both of Cu/Zn superoxide dismutase and ascorbate peroxidase. *China Biotech.*, **28**, 25–31.

58 Ahmad, R., Kim, Y.H., Kim, M.D., Kwon, S.Y., Cho, K., Lee, H.S. *et al.* (2010) Simultaneous expression of choline oxidase, superoxide dismutase and ascorbate peroxidase in potato plant chloroplasts provides synergistically enhanced protection against various abiotic stresses. *Physiol. Plant.*, **138** (4), 520–533.

59 Eltayeb, A.E., Yamamoto, S., Habora, M.E.E., Matsukubo, Y., Aono, M., Tsujimoto, H. *et al.* (2010) Greater protection against oxidative damages imposed by various environmental stresses in transgenic potato with higher level of reduced glutathione. *Breed. Sci.*, **60** (2), 101–109.

60 Foyer, C.H., Souriau, N., Perret, S., Lelandais, M., Kunert, K.J., Pruvost, C. *et al.* (1995) Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. *Plant Physiol.*, **109** (3), 1047–1057.

61 Rodrigues, S.M., Andrade, M.O., Gomes, A.P.S., DaMatta, F.M., Baracat-Pereira, M.C., and Fontes, E.P.B. (2006) *Arabidopsis* and tobacco plants ectopically expressing the soybean antiquitin-like ALDH7 gene display enhanced tolerance to drought, salinity, and oxidative stress. *J. Exp. Bot.*, **57** (9), 1909–1918

62 Struik, P.C. (2007) Responses of the potato plant to temperature, in *Potato Biology and Biotechnology* (eds D. Vreugdenhil, J. Bradshaw, C. Gebhardt,

F. Govers, D.K.L. Mackerron and M.A. Taylor *et al.*.), Elsevier Science BV, Amsterdam, p. 367.

63 Meehl, G.A., Stocker, T.F., Collins, W.D., Friedlingstein, P., Gaye, A.T., Gregory, J.M. *et al.* (2007) Global climate projections, in *Climate Change 2007: The Physical Science Basis* (eds S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt *et al.*.), Cambridge University Press, New York.

64 Kleinhenz, M.D. and Palta, J.P. (2002) Root zone calcium modulates the response of potato plants to heat stress. *Physiol. Plant.*, **115** (1), 111–118.

65 Arvin, M.J. and Donnelly, D.J. (2008) Screening potato cultivars and wild species to abiotic stresses using an electrolyte leakage bioassay. *J. Agri. Sci. Technol.*, **10** (1), 33–42.

66 Simon, G.A., Pinto, C.A.B.P., Lambert, E., and Andreu, M.A. (2009) Potato clones selection for early blight resistance and heat tolerance (Sele¸8cão de clones de batata resistentes à pinta preta e tolerantes ao calor). *Revista Ceres*, **56** (1), 31–37.

67 Mienie, A. and Ronde, J.A. (2008) A comparison of drought stress and heat stress in the leaves and tubers of 12 potato cultivars. *S. Afr. J. Sci.*, **104** (3), 156–159.

68 Kumar, R. and Kang, G.S. (2007) Analysis of variance and combining ability of yield components of potato (*Solanum tuberosum*) under early planting heat stress conditions. *Indian J. Agric. Sci.*, **77** (8), 523–525.

69 Ahn, Y.J., Claussen, K., and Zimmerman, J.L. (2004) Genotypic differences in the heat-shock response and thermotolerance in four potato cultivars. *Plant Sci.*, **166** (4), 901–911.

70 Thompson, A.L., Love, S.L., Sowokinos, J.R., Thornton, M.K., and Shock, C.C. (2008) Review of the sugar end disorder in potato (*Solanum tuberosum*, L.). *Am. J. Potato Res.*, **85** (5), 375–386.

71 Yencho, G.C., Sterrett, S.B.R., Haynes, K.G., and McCord, P.H. (2008) Internal heat necrosis of potato: a review [electronic resource]. *Am. J. Potato Res.*, **85** (1), 69–76.

72 Ginzberg, I., Barel, G., Ophir, R., Tzin, E., Tanami, Z., Muddarangappa, T. *et al.* (2009) Transcriptomic profiling of heat-stress response in potato periderm. *J. Exp. Bot.*, **60** (15), 4411–4421.

73 Ryu, S.H., Kim, Y.H., Kim, C.Y., Park, S.Y., Kwon, S.Y., Lee, H.S. *et al.* (2009) Molecular characterization of the sweet potato peroxidase SWPA4 promoter which responds to abiotic stresses and pathogen infection. *Physiol. Plant.*, **135** (4), 390–399.

74 Tang, L., Kwon, S., Kim, S., Kim, J., Choi, J., Cho, K. *et al.* (2006) Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. *Plant Cell Rep.*, **25** (12), 1380–1386.

75 Rensink, W., Hart, A., Liu, J., Ouyang, S., Zismann, V., and Buell, C.R. (2005) Analyzing the potato abiotic stress transcriptome using expressed sequence tags. *Genome*, **48** (4), 598–605.

76 Rensink, W.A., Iobst, S., Hart, A., Stegalkina, S., Liu, J., and Buell, C.R. (2005) Gene expression profiling of potato responses to cold, heat, and salt stress. *Funct. Integr. Genomics*, **5** (4), 201–207.

77 Oufir, M., Legay, S., Nicot, N., Moer, K., Hoffmann, L., Renaut, J. *et al.* (2008) Gene expression in potato during cold exposure: changes in carbohydrate and polyamine metabolisms. *Plant Sci.*, **175** (6), 839–852.

78 Badea, C. and Basu, S.K. (2009) The effect of low temperature on metabolism of membrane lipids in plants and associated gene expression. *Plant Omics*, **2** (2), 78–84.

79 Palta, J.P., Whitaker, B.D., and Weiss, L.S. (1993) Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of *Solanum* species. *Plant Physiol.*, **103** (3), 793–803.

80 Vega, S.E., Rio, A.H., Bamberg, J.B., and Palta, J.P. (2004) Evidence for the up-regulation of stearoyl-ACP (Δ9) desaturase gene expression during cold

acclimation. *Am. J. Potato Res.*, **81** (2), 125–135.

81 Amiri, R.M., Yur'eva, N.O., Shimshilashvili, K.R., Goldenkova-Pavlova, I., Pchelkin, V.P., Kuznitsova, E.I. *et al.* (2010) Expression of acyl-lipid Δ12-desaturase gene in prokaryotic and eukaryotic cells and its effect on cold stress tolerance of potato. *J. Integr. Plant Biol.*, **52** (3), 289–297.

82 Maali-Amiri, R., Trunova, T.I., Deryabin, A.N., Nosov, A.M., Los, D.A., Vereshchagin, A.G. *et al.* (2007) Lipid fatty acid composition of potato plants transformed with the Δ12-desaturase gene from cyanobacterium [electronic resource]. *Russ. J. Plant Physiol.*, **54** (5), 600–606.

83 Palta, J.P., Weiss, L.S., and Whitaker, B.D. (1993) Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of *Solanum* species. *Plant Physiol.*, **103** (3), 793–803.

84 Kielbowicz-Matuk, A., Rey, P., and Rorat, T. (2008) The organ-dependent abundance of a Solanum lipid transfer protein is up-regulated upon osmotic constraints and associated with cold acclimation ability. *J. Exp. Bot.*, **59** (8), 2191–2203.

85 Pramanik, M.H.R. and Imai, R. (2005) Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. *Plant Mol. Biol.*, **58** (6), 751–762.

86 Wi, S.J., Kim, W.T., and Park, K.Y. (2006) Overexpression of carnation *S*-adenosylmethionine decarboxylase gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants. *Plant Cell Rep.*, **25** (10), 1111–1121.

87 Akiyama, T. and Jin, S. (2007) Molecular cloning and characterization of an arginine decarboxylase gene up-regulated by chilling stress in rice seedlings. *J. Plant Physiol.*, **164** (5), 645–654.

88 Rorat, T., Irzykowski, W., Berbezy, P., and Grygorowicz, W.J. (1998) Isolation and expression of cold specific genes in potato (*Solanum sogarandinum*). *Plant Sci.*, **133** (1), 57–67.

89 Deryabin, A.N., Dubinina, I.M., Burakhanova, E.A., Astakhova, N.V., Sabel'Nikova, E.P., Sinkevich, M.S. *et al.* (2004) Cold tolerance of potato plants transformed with yeast invertase gene. *Acta Agrobot.*, **57** (1), 31–39.

90 Deryabin, A.N., Dubinina, I.M., Burakhanova, E.A., Astakhova, N.V., Sabel'nikova, E.P., and Trunova, T.I. (2005) Influence of yeast-derived invertase gene expression in potato plants on membrane lipid peroxidation at low temperature. *J. Therm. Biol.*, **30** (1), 73–77.

91 Massarelli, I., Grillo, S., Costa, A., and Leone, A. (2002) Differential expression of potato calmodulin genes by cold, heat and salt stress. *J. Genet. Breed.*, **56** (4), 331–337.

92 Mora-Herrera, M. and López-Delgado, H.A. (2007) Freezing tolerance and antioxidant activity in potato microplants induced by abscisic acid treatment. *Am. J. Potato Res.*, **84** (6), 467–475.

93 Lopez-Delgado, H., Scott, I.M., and Mora-Herrera, M. (2007) Stress and antistress effects of salicylic acid and acetyl salicylic acid on potato culture technology, in *Salicylic Acid: A Plant Hormone*, Springer.

94 Mora-Herrera, M., López-Delgado, H., Castillo-Morales, A., and Foyer, C.H. (2005) Salicylic acid and H_2O_2 function by independent pathways in the induction of freezing tolerance in potato. *Physiol. Plant.*, **125** (4), 430–440.

95 Lukatkin, A.S. (2002) Contribution of oxidative stress to the development of cold-induced damage to leaves of chilling-sensitive plants: 1. Reactive oxygen species formation during plant chilling. *Russ. J. Plant Physiol.*, **49** (5), 622–627.

96 Seppänen, M.M. and Coleman, G.D. (2003) Characterization of genotypic variation in stress gene expression and photosynthetic parameters in potato. *Plant Cell Environ.*, **26** (3), 401–410.

97 Sin'kevich, M.S., Deryabin, A.N., and Trunova, T.I. (2009) Characteristics of

oxidative stress in potato plants with modified carbohydrate metabolism. *Russ. J. Plant Physiol.*, **56** (2), 168–174.

98 Sinkevich, M.S., Naraykina, N.V., and Trunova, T.I. (2010) Involvement of sugars in the antioxidant defense against paraquat-induced oxidative stress in potato transformed with yeast invertase gene. *Dokl. Biol. Sci.*, **434** (1), 338–340.

99 Pruvot, G., Massimino, J., Peltier, G., and Rey, P. (1996) Effects of low temperature, high salinity and exogenous ABA on the synthesis of two chloroplastic drought-induced proteins in *Solanum tuberosum*. *Physiol. Plant.*, **97** (1), 123–131.

100 Massarelli, I., Cioffi, R., Batelli, G., Palma, M., Costa, A., Grillo, S. *et al.* (2006) Functional screening of plant stress-related cDNAs by random over-expression in *Escherichia coli*. *Plant Sci.*, **170** (4), 880–888.

101 Rorat, T., Yin, Z., Rey, P., Wojtowicz, B., Szabala, B.M., and Grygorowicz, W.J. (2006) Expression of SK3-type dehydrin in transporting organs is associated with cold acclimation in *Solanum* species [electronic resource]. *Planta*, **224** (1), 205–221.

102 Wallis, J.G., HongYu, W., and Guerra, D.J. (1997) Expression of a synthetic antifreeze protein in potato reduces electrolyte release at freezing temperatures. *Plant Mol. Biol.*, **35** (3), 323–330.

103 JungWon, Y., JaeHeung, J., Doil, C., SoYoung, Y., Hyouk, J., and HyunSoon, K. (2008) Ectopic expression of pepper CaPF1 in potato enhances multiple stresses tolerance and delays initiation of *in vitro* tuberization. *Planta*, **228** (4), 701–708.

104 Lee, H.E., Shin, D., Park, S.R., Han, S.E., Jeong, M.J., Kwon, T.R. *et al.* (2007) Ethylene responsive element binding protein 1 (StEREBP1) from *Solanum tuberosum* increases tolerance to abiotic stress in transgenic potato plants. *Biochem. Biophys. Res. Commun.*, **353** (4), 863–868.

105 Behnam, B., Kikuchi, A., Celebi-Toprak, F., Kasuga, M., Yamaguchi-Shinozaki, K., and Watanabe, K.N. (2007) Arabidopsis rd29A::DREB1A enhances freezing tolerance in transgenic potato. *Plant Cell Rep.*, **26** (8), 1275–1282.

106 Pino, M.T., Skinner, J.S., EungJun, P., Jeknic′, Z., Hayes, P.M., Thomashow, M.F. *et al.* (2007) Use of a stress inducible promoter to drive ectopic AtCBF expression improves potato freezing tolerance while minimizing negative effects on tuber yield. *Plant Biotechnol. J.*, **5** (5), 591–604.

107 Pino, M.T., Skinner, J.S., Jeknic′, Z., Hayes, P.M., Soeldner, A.H., Thomashow, M.F. *et al.* (2008) Ectopic AtCBF1 over-expression enhances freezing tolerance and induces cold acclimation-associated physiological modifications in potato. *Plant Cell Environ.*, **31** (4), 393–406.

108 Nie, X., Tai, G.C.C., and Singh, R.P. (2002) Molecular characterization and expression analysis of 1-aminocyclopropane-1-carboxylate oxidase homologs from potato under abiotic and biotic stresses. *Genome*, **45** (5), 905–913.

109 Renaut, J., Planchon, S., Oufir, M., Hausman, J.F., Hoffmann, L., and Evers, D. (2009) Identification of proteins from potato leaves submitted to chilling temperature, in *Plant Cold Hardiness: From the Laboratory to the Field*, CABI Publishing.

110 Sowokinos, J.R. (2001) Biochemical and molecular control of cold-induced sweetening in potatoes. *Am. J. Potato Res.*, **78** (3), 221–236.

111 Bhaskar, P.B., Wu, L., Busse, J.S., Whitty, B.R., Hamernik, A.J., Jansky, S.H. *et al.* (2010) Suppression of the vacuolar invertase gene prevents cold-induced sweetening in potato. *Plant Physiol.*, **154** (2), 939–948.

112 Krause, K., Hill, L., Reimholz, R., Hamborg Nielsen, T., Sonnewald, U., and Stitt, M. (1998) Sucrose metabolism in cold-stored potato tubers with decreased expression of sucrose phosphate synthase. *Plant Cell Environ.*, **21** (3), 285–299.

113 Macrae, E. and Lunn, J. (2006) *Control of Sucrose Biosynthesis*, Blackwell Publishing Ltd.

114 Zrenner, R., Schüler, K., and Sonnewald, U. (1996) Soluble acid invertase

determines the hexose-to-sucrose ratio in cold-stored potato tubers. *Planta*, **198** (2), 246–252.

115 Flinn, B., Rothwell, C., Griffiths, R., Lague, M., DeKoeyer, D., Sardana, R. *et al.* (2005) Potato expressed sequence tag generation and analysis using standard and unique cDNA libraries. *Plant Mol. Biol.*, **59** (3), 407–433.

116 Shuai, C., Hajirezaei, M.R., Zanor, M.I., Hornyik, C., Debast, S., Lacomme, C. *et al.* (2008) RNA interference-mediated repression of sucrose-phosphatase in transgenic potato tubers (*Solanum tuberosum*) strongly affects the hexose-to-sucrose ratio upon cold storage with only minor effects on total soluble carbohydrate accumulation. *Plant Cell Environ.*, **31** (1), 165–176.

117 Hamernik, A.J., Jansky, S.H., and Hanneman, R.E.J. (2009) Introgression of wild species germplasm with extreme resistance to cold sweetening into the cultivated potato [electronic resource]. *Crop Sci.*, **49** (2), 529–542.

118 Greiner, S., Rausch, T., Sonnewald, U., and Herbers, K. (1999) Ectopic expression of a tobacco invertase inhibitor homolog prevents cold-induced sweetening of potato tubers. *Nat. Biotechnol.*, **17** (7), 708–711.

119 ShanHan, C., Jun, L., CongHua, X., BoTao, S., and JingCai, L. (2006) Role of tobacco vacuolar invertase regulated by patatin promoter in resistance of potato tubers to cold-sweetening. *J. Agric. Biotechnol.*, **14** (5), 716–720.

120 Agarwal, S., Chakrabarti, S.K., Misra, S., Chimote, V.P., Pattanayak, D., and Naik, P.S. (2003) A biotechnological approach for reduction of cold-induced sweetening in potato tubers. *J. Indian Potato Assoc.*, **30** (1), 39–40.

121 Chi, Z., CongHua, X., BoTao, S., Xun, L., and Jun, L. (2008) RNAi effects on regulation of endogenous acid invertase activity in potato (*Solanum tuberosum* L.) tubers. *Chin. J. Agric. Biotechnol.*, **5** (2), 107–112.

122 Chi, Z., CongHua, X., Jun, L., BoTao, S., and Xun, L. (2008) Effects of RNAi on regulation of endogenous acid invertase activity in potato (*Solanum tuberosum* L.) tubers. *J. Agric. Biotechnol.*, **16** (1), 108–113.

123 Donnelly, D.J., Prasher, S.O., and Patel, R.M. (2007) Towards the development of salt-tolerant potato, in *Potato Biology and Biotechnology* (eds D. Vreugdenhil, J. Bradshaw, C. Gebhardt, F. Govers, D.K.L. Mackerron, M.A. Taylor *et al.*), Elsevier Science BV, Amsterdam, p. 415.

124 Legay, S., Lamoureux, D., Hausman, J.F., Hoffmann, L., and Evers, D. (2009) Monitoring gene expression of potato under salinity using cDNA microarrays. *Plant Cell Rep.*, **28** (12), 1799–1816.

125 Aghaei, K., Ehsanpour, A.A., and Komatsu, S. (2008) Proteome analysis of potato under salt stress. *J. Proteome Res.*, **7** (11), 4858–4868.

126 Tian, W., ZhenDong, T., Jun, L., and CongHua, X. (2009) A novel leucine-rich repeat receptor-like kinase gene in potato, StLRPK1, is involved in response to diverse stresses. *Mol. Biol. Rep.*, **36** (8), 2365–2374.

127 Swiedrych, A., Lorenc-Kukula, K., Skirycz, A., and Szopa, J. (2004) The catecholamine biosynthesis route in potato is affected by stress. *Plant Physiol. Biochem.*, **42** (7–8), 593–600.

128 Ni, X., Li, J., Shi, X., Xie, C., Tian, Z., Liu, J. *et al.* (2010) StPUB17, a novel potato UND/PUB/ARM repeat type gene, is associated with late blight resistance and NaCl stress [electronic resource]. *Plant Sci.*, **178** (2), 158–169.

129 Guo, F.Q., Okamoto, M., and Crawford, N.M. (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science*, **302** (5642), 100–103.

130 Zhao, M., Tian, Q., and Zhang, W. (2007) Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in *Arabidopsis*. *Plant Physiol.*, **144** (1), 206–217.

131 Vitecek, J., Reinohl, V., and Jones, R.L. (2008) Measuring NO production by plant tissues and suspension cultured cells. *Mol. Plant*, **1** (2), 270–284.

132 Zhang, B., Wang, H.Q., Liu, B.L., Liu, J., Wang, X., Liu, Q. *et al.* (2010) A potato NOA gene increased salinity tolerance in *Arabidopsis thaliana*. *Afr. J. Biotechnol.*, **9** (36), 5869–5878.

133 Hmida-Sayari, A., Gargouri-Bouzid, R., Bidani, A., Jaoua, L., Savouré, A., and Jaoua, S. (2005) Overexpression of Δ1-pyrroline-5-carboxylate synthetase increases proline production and confers salt tolerance in transgenic potato plants. *Plant Sci.*, **169** (4), 746–752.

134 Rhodes, D. and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **44** (1), 357–384.

135 Papageorgiou, G.C. and Murata, N. (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. *Photosyn. Res.*, **44** (3), 243–252.

136 Chen, T.H. and Murata, N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.*, **5** (3), 250–257.

137 Ahmad, R., Kim, M., Back, K., Kim, H., Lee, H., Kwon, S. *et al.* (2008) Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses. *Plant Cell Rep.*, **27** (4), 687–698.

138 Ning, Z., HuaiJun, S., Liang, L., Tao, Y., ChunFeng, Z., and Di, W. (2009) Drought and salinity tolerance in transgenic potato expressing the betaine aldehyde dehydrogenase gene. *Acta Agron. Sin.*, **35** (6), 1146–1150.

139 Shaterian, J., Georges, F., Hussain, A., Waterer, D., Jong, H., and Tanino, K.K. (2005) Root to shoot communication and abscisic acid in calreticulin (CR) gene expression and salt-stress tolerance in grafted diploid potato clones. *Environ. Exp. Bot.*, **53** (3), 323–332.

140 Daneshmand, F., Arvin, M.J., and Kalantari, K.M. (2010) Acetylsalicylic acid (Aspirin) induces salinity and osmotic tolerance in *Solanum acaule in vitro*. *Agrochimica*, **54** (1), 52–64.

141 JunLian, Z., Li, W., Di, W., JinWen, Z., and ZhengHua, C. (2007) Generation of transgenic potato plants harboring AtNHX1 gene mediated by *Agrobacterium tumefaciens*. *Acta Agron. Sin.*, **33** (7), 1067–1072.

142 Bayat, F., Shiran, B., Belyaev, D.V., Yur'eva, N.O., Sobol'kova, G.I., Alizadeh, H. *et al.* (2010) Potato plants bearing a vacuolar Na^+/H^+ antiporter HvNHX2 from barley are characterized by improved salt tolerance. *Russ. J. Plant Physiol.*, **57** (5), 696–706.

143 Lovas, A., Banfalvi, Z., Szabo, L., and Bimbo, A. (2003) Antisense repression of StubGAL83 affects root and tuber development in potato. *Plant J.*, **33** (1), 139–147.

144 Jeong, M.J., Park, S.C., and Byun, M.O. (2001) Improvement of salt tolerance in transgenic potato plants by glyceraldehyde-3 phosphate dehydrogenase gene transfer. *Mol. Cells*, **12** (2), 185–189.

145 Teixeira, J. and Fidalgo, F. (2009) Salt stress affects glutamine synthetase activity and mRNA accumulation on potato plants in an organ-dependent manner. *Plant Physiol. Biochem.*, **47** (9), 807–813.

146 Liu, D., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A. (1994) Osmotin overexpression in potato delays development of disease symptoms. *Proc. Natl. Acad. Sci. USA*, **91** (5), 1888–1892.

147 Li, R., Wu, N., Fan, Y., and Song, B. (1999) Transgenic potato plants expressing osmotin gene inhibits fungal development in inoculated leaves. *Chin. J. Biotechnol.*, **15** (2), 71–75.

148 Evers, D., Overney, S., Simon, P., Greppin, H., and Hausman, J.F. (1999) Salt tolerance of *Solanum tuberosum* L. overexpressing an heterologous osmotin-like protein. *Biol. Plant.*, **42** (1), 105–112.

149 El-Banna, A., Hajirezaei, M.R., Wissing, J., Ali, Z., Vaas, L., Heine-Dobbernack, E. *et al.* (2010) Over-expression of PR-10a leads to increased salt and osmotic tolerance in potato cell cultures. *J. Biotechnol.*, **150** (3), 277–287.

150 Celebi-Toprak, F., Behnam, B., Serrano, G., Kasuga, M., Yamaguchi-Shinozaki, K., Naka, H. *et al.* (2005) Tolerance to salt stress of the transgenic tetrasomic tetraploid potato, *Solanum tuberosum* cv. Desiree appears to be induced by the DREB1A gene and rd29A promoter of *Arabidopsis thaliana*. *Breed. Sci.*, **55** (3), 311–319.

151 HuiZhen, L., ZhiJun, Z., Ling, X., WenJian, S., and WeiJun, Z. (2006) Effect of salinity on chlorophyll contents, proline accumulation and antioxidant enzyme activities of plantlets *in vitro* in potato. *J. Zhejiang Univ. (Agri. Life Sci.)*, **32** (3), 300–306.

152 RuiJiu, Z., GuoBin, S., MeiLian, M., FuYi, M., HaiLong, L., and JunXiu, G. (2007) Effects of NaCl stress on antioxidant defence system of potato. *Chinese Potato J.*, **21** (1), 11–14.

153 Fidalgo, F., Santos, A., Santos, I., and Salema, R. (2004) Effects of long-term salt stress on antioxidant defence systems, leaf water relations and chloroplast ultrastructure of potato plants. *Ann. Appl. Biol.*, **145** (2), 185–192.

154 Aghaei, K., Ehsanpour, A.A., and Komatsu, S. (2009) Potato responds to salt stress by increased activity of antioxidant enzymes. *J. Integr. Plant Biol.*, **51** (12), 1095–1103.

155 Sajid, Z.A. and Aftab, F. (2009) Amelioration of salinity tolerance in Solanum tuberosum L. by exogenous application of ascorbic acid. *In Vitro Cell. Dev. Biol. Plant*, **45** (5), 540–549

156 M'Hamdi, M., Bettaieb, T., Harbaoui, Y., Mougou, A.A., and Jardin, P. (2009) Insight into the role of catalases in salt stress in potato (*Solanum tuberosum* L.). *Biotechnol. Agron. Soc. Environ.*, **13** (3), 373–379.

157 Hemavathi, Upadhyaya, C.P., Akula, N., Young, K.E., Chun, S.C., Kim, D.H. *et al.* (2010) Enhanced ascorbic acid accumulation in transgenic potato confers tolerance to various abiotic stresses. *Biotechnol. Lett.*, **32** (2), 321–330.

158 Lulai, E.C. (2007) Skin-set, wound healing, and related defects, in *Potato Biology and Biotechnology* (eds D. Vreugdenhil, J. Bradshaw, C. Gebhardt, F. Govers, D.K.L. Mackerron, and M.A. Taylor *et al.*.), Elsevier Science B.V., Amsterdam, p. 471.

159 Lulai, E.C. and Suttle, J.C. (2009) Signals involved in tuber wound-healing [electronic resource]. *Plant Signal. Behav.*, **4** (7), 620–622.

160 Kumar, G.N.M., Knowles, N.R., Suttle, J.C., and Lulai, E.C. (2010) Age-induced loss of wound-healing ability in potato tubers is partly regulated by ABA. *Planta*, **232** (6), 1433–1445.

161 Bernards, M.A. (2002) Demystifying suberin. *Can. J. Bot.*, **80**, 227–240.

162 Chaves, I., Pinheiro, C., Paiva, J.A.P., Planchon, S., Sergeant, K., Renaut, J. *et al.* (2009) Proteomic evaluation of wound-healing processes in potato (*Solanum tuberosum* L.) tuber tissue. *Proteomics*, **9** (17), 4154–4175.

163 Kumar, G.N.M. and Knowles, N.R. (2003) Wound-induced superoxide production and PAL activity decline with potato tuber age and wound healing ability. *Physiol. Plant.*, **117** (1), 108–117.

164 Lulai, E.C., Suttle, J.C., and Pederson, S.M. (2008) Regulatory involvement of abscisic acid in potato tuber wound-healing. *J. Exp. Bot.*, **59** (6), 1175–1186.

165 Yang, W. and Bernards, M. (2007) Metabolite profiling of potato (*Solanum tuberosum* L.) tubers during wound-induced suberization. *Metabolomics*, **3** (2), 147–159.

166 Bernards, M.A., Summerhurst, D.K., and Razem, F.A. (2004) Oxidases, peroxidases and hydrogen peroxide: the suberin connection. *Phytochem. Rev.*, **3** (1), 113–126.

167 Malmberg, A. (1984) N-Feruloylputrescine in infected potato tubers. *Acta Chem. Scand. B*, **38** (2), 153–155.

168 Razem, F.A. and Bernards, M.A. (2002) Hydrogen peroxide is required for poly(phenolic) domain formation during wound-induced suberization. *J. Agric. Food Chem.*, **50** (5), 1009–1015.

169 Korth, K.L. and Dixon, R.A. (1997) Evidence for chewing insect-specific molecular events distinct from a general wound response in leaves. *Plant Physiol.*, **115** (4), 1299–1305.

170 Bricchi, I., Boland, W., Maffei, M.E., Mith©œfer, A., Leitner, M., and Foti, M. (2010) Robotic mechanical wounding (MecWorm) versus herbivore-induced responses: early signaling and volatile emission in Lima bean (*Phaseolus lunatus* L.) [electronic resource]. *Planta*, **232** (3), 719–729.

171 Turra, D., Bellin, D., Lorito, M., and Gebhardt, C. (2009) Genotype-dependent expression of specific members of potato protease inhibitor gene families in different tissues and in response to wounding and nematode infection. *J. Plant Physiol.*, **166** (7), 762–774.

172 Heibges, A., Glaczinski, H., Ballvora, A., Salamini, F., and Gebhardt, C. (2003) Structural diversity and organization of three gene families for Kunitz-type enzyme inhibitors from potato tubers (*Solanum tuberosum*L.). *Mol. Genet. Genomics*, **269** (4), 526–534.

173 Ussuf, K.K., Laxmi, N.H., and Mitra, R. (2001) Proteinase inhibitors: plant-derived genes of insecticidal protein for developing insect-resistant transgenic plants. *Curr. Sci.*, **80** (7), 847–853.

174 Cai, D., Thurau, T., Tian, Y., Lange, T., Yeh, K., and Jung, C. (2003) Sporamin-mediated resistance to beet cyst nematodes (*Heterodera schachtii* Schm.) is dependent on trypsin inhibitory activity in sugar beet (*Beta vulgaris* L.) hairy roots. *Plant Mol. Biol.*, **51** (6), 839–849.

175 Wang-Pruski, G. and Nowak, J. (2004) Potato after-cooking darkening. *Am. J. Potato Res.*, **81** (1), 7–16.

176 Murphy, J.P., Kong, F., Pinto, D.M., and Wang-Pruski, G. (2010) Relative quantitative proteomic analysis reveals wound response proteins correlated with after-cooking darkening. *Proteomics*, **10** (23), 4258–4269.

177 Rommens, C.M. (2007) Intragenic crop improvement: combining the benefits of traditional breeding and genetic engineering. *J. Agric. Food Chem.*, **55** (11), 4281–4288.

178 Rommens, C.M., Haring, M.A., Swords, K., Davies, H.V., and Belknap, W.R. (2007) The intragenic approach as a new extension to traditional plant breeding. *Trends Plant Sci.*, **12** (9), 397–403.

45
Potato: Production Strategies under Abiotic Stress

Joginder Singh Minhas

Potato production is rapidly expanding in tropical and sub-tropical environments. The population density in these areas is high and potato with its high productivity of edible energy per unit area and time has the potential to alleviate hunger and malnutrition. However, the crop is exposed to various kinds of abiotic stresses like drought, heat, salinity etc. in these environments which are important limiting factors for potato productivity. The average tuber yield in these areas is less than half compared to temperate climates. With increased human activity impacting climate change, these stresses are likely to be experienced in higher magnitude and more areas. Genetic variability exists for tolerance to these stresses in potato and related species germplasm and can be exploited for developing abiotic stress tolerant varieties. Moreover, it is important to understand stress tolerance mechanisms operating in different plant species, and utilize our knowledge of agronomy, physiology, genetics and molecular biology to develop new genotypes capable of giving good yields under stressful environments.

45.1
Introduction

Although cultivated potatoes originated in highlands of South America, full potential of the crop was exploited in the temperate countries through organized breeding work. From these countries, potato was introduced to the tropical and subtropical areas of the world. As of now, potato is one of the most important food crops, both in developed and in developing countries. Owing to high protein–calorie ratio (17 g protein:1000 kcal) and short vegetative cycle, potato yields substantially more edible energy, protein, and dry matter per unit area and time than many other crops. In 2005, potato production by the developing countries overtook production by the developed countries. Most of these countries lie in tropical and subtropical zones of the world and are prone to various abiotic stresses such as drought, salinity, and high temperature. Abiotic stresses reduce the potential crop yields to a large extent and

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present a major challenge to sustainable food production. Water is recognized as the most crucial natural resource for agricultural productivity. Water stress related problems are aggravating all over the world since droughts are becoming a common occurrence in rain-fed agriculture, more so, because of global weather changes. Scanty rainfall or inadequate irrigation creates drought-like situation resulting in reduced water availability to the crops. Loss of water due to high temperature also contributes to drought and leads to symptoms typical of dehydration or desiccation. Physiological drought is caused by high osmolarity (due to excessive salinity) of the soil because of which plants cannot utilize water even if available. Salinity directly affects ionic balance in the cells resulting in reduced growth and dry matter production. Temperature has profound effect on plant growth and development. Low temperatures lead to reduced photosynthesis and frost injury. High temperature has direct and indirect effects that reduce plant productivity. Direct effects are reduced photosynthesis and increased respiration leaving little photosynthate for growth and development. Indirectly high temperature disturbs partitioning of sugars among different organs affecting yield; for example, in potato, translocation of sugars is diverted to aboveground parts leading to severe yield reduction even at mild night temperatures.

Physiologically, stress tolerance can be defined in many ways like ability of the plant to survive under severe stress through osmotic adjustment and reduced metabolism, percentage yield reduction under stress compared to control, seed setting under stress, and so on, but from agronomic point of view, total commercial crop yield under stress is the only criterion. Therefore, the aim of the agricultural scientist is to develop stress-tolerant varieties and agrotechniques to maximize crop productivity under stressful environmental conditions.

45.2 Drought Stress

Drought is considered to be the major limiting factor for yield in the world potato production [1] influencing negatively not only yield but also tuber quality. Insufficient water supply may occur almost anywhere where potatoes are grown. In arid regions (e.g., subtropics), where potato production is possible only with irrigation, short periods of drought often arise because of inadequate irrigation techniques or shortage of water. Even with good irrigation practices, water stress may occur because of high transpiration rates, especially during mid-day, when root system cannot completely meet the water requirements of the plant, leading to increased water potential and consequent reduction in the rate of photosynthesis [2]. In the temperate climatic zones, both short and long periods of drought may occur almost every year due to irregular rainfall, particularly on soils with low water holding capacity. Taking into account production conditions and the present yield levels, it is estimated that the average potato yield in the world could be increased by at least 50% if the water supply to the crop could be optimized. Introduction of drought tolerance in potato through breeding and biotechnological means should therefore receive high priority.

45.2.1
Potato Growth and Production

Drought may affect potato growth and production in three ways: (1) by reducing the amount of productive foliage, (2) by decreasing the rate of photosynthesis per unit of leaf area, and (3) by shortening the vegetative growth period. Drought after planting may delay or even inhibit germination. Drought after planting is generally experienced by the potato crop under rain-fed conditions. Bansal and Nagarajan [3] found that water stress caused reduction in leaf growth in all the eight cultivars tested by them, although the extent of reduction varied within the varieties. Even mild water stress of −3 to −5 bars greatly reduced leaf expansion in potatoes [4]. Similar results on reduction of leaf growth under water stress were obtained by other workers [5–7]. Insufficient water supply in the period between the emergence and the beginning of tuber bulking may therefore lead to a limited growth rate of foliage and to small leaves and small plants. As a consequence, soil cover with green foliage will often be incomplete and yields will be below optimum. Decline in the rate of photosynthesis is fast and substantial even at relatively low water potentials of −3 to −5 bars [8, 9]. Even in the irrigated crop, plants experience water stress during the mid-day as the roots are not able to fully meet the transpirational demands of the plants. Mid-day depression in the rate of photosynthesis in well-irrigated crop was reported by Minhas and Sukumaran [2]. Plants respond to water stress by closing their stomata, thus shutting out the supply of CO_2. Sugar concentration within the leaf tissue increases to increase the osmotic potential of the plant, thus leading to feedback inhibition of photosynthesis [10]. Sensitivity of the potato crop to water stress varies with the developmental stage of the crop. Various authors have defined these stages as per their convenience; however, the stage between stolon initiation and early tuber development is the most sensitive, and stress at this stage causes maximum reduction in tuber yield [11–14]. Water stress during the tuber bulking stage caused a reduction in the leaf expansion rate, but to a lesser extent, compared to plants before tuber initiation. The presence of the tubers probably increased the water capacitance of the plants [10, 15] leading to reduced effect of water stress. Apart from reduction in leaf growth, water stress during tuber bulking stage accelerates plant senescence resulting in decreased LAI. At first, lower leaves start to wilt and drop off, while drought simultaneously inhibits the development of new leaves [16].

45.2.2
Drought and Tuber Quality

Drought stress also affects tuber quality characteristics such as shape, dry matter content, and reducing sugar content. Tuber shape defects such as dumbbell-shaped, knobby, or pointed end tubers are caused by short periods of drought during the tuber bulking stage. Misshapen tubers can also occur due to secondary growth, which is especially likely to occur in dry soils where temperature can go high [17]. This phenomenon may also result in poor cooking quality (glassiness) in some of the

tubers or jelly end or translucent end tubers. All these tubers have high content of reducing sugars, which makes them unfit for the processing industry. Tubers of water-stressed plants often have higher content of total sugars than well-irrigated plants [18]. Some studies have shown that the dry matter and starch content of potato tubers grown under low soil moisture was higher than the well-watered plants, thus improving the quality [19–21]. Other studies have shown that dry matter declines upon exposure to drought [22]; however, there were varietal differences for this character [18]. Tuber starch content has been reported to increase under drought stress [5].

45.2.3 Coping with Drought Stress

With the spread of potato cultivation to tropical and subtropical areas, the crop is likely to be exposed to increasing incidences of drought stress. Therefore, to cope with it, the first line of defense is the development of drought-tolerant varieties. Genetic variability exists in the potato germplasm, varieties, and wild species for tuber yield under drought stress [23–25]. Newer techniques such as heavy carbon isotope (^{13}C) discrimination based on $\Delta^{13}C$ values indicate water use efficiency of the plant. Screening of potato germplasm for $\Delta^{13}C$ showed good genetic variability for this character and can be utilized for breeding drought-tolerant varieties [26]. Screening techniques have also been developed by various workers on the basis of leaf extension after relief of stress [3], root mass in *in vitro* plantlets [27, 28], isotope discrimination ratio [29], and field screening using line source principle [30]. Using these techniques, some of the drought-tolerant varieties have already been developed/screened [18, 23, 24, 31].

Along with developing tolerant varieties, drought stress can be managed to some extent by various agronomic, chemical, and biological means. Soil water stress combined with higher atmospheric evaporative demand leads to severe stress affected yield losses [32]; therefore, crop under limited water supply can be grown when atmospheric evaporative demand is low. Mulching with agricultural waste during periods of drought helps in conservation of water, better crop stand and yields [33]. Plastic mulch on the ridges helps in rainwater harvesting and conservation between the ridges leading to better tuber yields [34]. Potassium application to the soil [35, 36] and spray of potassium humate [37] improves crop performance under drought stress. The use of gel polymers in the soil under water-limiting conditions improves water availability to the crop and tuber yield [38]. Some rhizobacteria contain the enzyme 1-aminocyclopropane-1-carboxylase (ACC) deaminase that degrades the ethylene precursor ACC and promotes plant growth, particularly under unfavorable environmental conditions such as drought. These bacteria can attenuate the growth inhibition caused by water deficit [39]. Better tuber yield can be obtained under water-limiting conditions by using drip irrigation combined with appropriate placement of drip tapes. While Onder *et al.* [40] found surface and subsurface drip equally effective, Patel and Rajput [41] reported a distinct advantage of subsurface drip irrigation at 10 cm for obtaining maximum tuber yield

at 100 and 80% irrigation levels. A combination of drought-tolerant varieties coupled with modern water saving irrigation techniques can be used to successfully produce potatoes in arid and semiarid zones of the world.

45.3 Heat Stress

Potato originated and evolved in the tropical highlands of Andes and hence prefers cool climate (17 °C) for optimum tuber yield [42]. Higher temperature may inhibit yield by overall reduction of plant development due to heat stress or by reduced partitioning of assimilates to tubers. Minimum night temperature is very important for potato crop. Whether or not potato will tuberize depends largely on the minimum night temperature and not on the average daily temperature. Tuberization is reduced at night temperatures above 18 °C and there may not be any tuberization at night temperature of 25 °C and above, even though potato plants can tolerate day temperature of about 35 °C without much deleterious effect. High temperature induces development of plants with thin stems, small leaves, and long stolons, increase in the number of internodes, inhibition of tuber development and a decrease in the ratio of tuber fresh weight to total fresh weight [43–46]. Heat stress affects many plant processes, and some of them are discussed in the following sections.

45.3.1 Photosynthesis and Respiration

Optimum temperature for dark respiration is 16–20 °C [47] and for photosynthesis it is 24–28 °C [48]. Higher temperature increases the rate of dark respiration and reduces the rate of photosynthesis in plants. Bushnell [42] measured the rate of night respiration in potato plants at different temperatures and found doubling of respiration with every 10 °C increase in temperature. So, as the temperature increases, more and more carbohydrates are used up as respiratory substrate and less and less are synthesized and are available for translocation to the tubers. At certain temperature (30 °C according to Burton [49]), there is no net assimilation. However, chlorophyll fluorescence studies showed that photosynthetic apparatus in potato is stable up to 38 °C, but beyond that there is a drastic reduction in photosynthetic efficiency [50].

45.3.2 Tuberization

The most prominent effect of high temperature is on the partitioning of assimilated carbon between leaves and tubers. The inhibition of tuberization at high temperature has often been demonstrated since the early study by Bushnell [42]. Gregory [51] found that tubers were initiated under short days over a long range of day tempera-

tures, but initiation was depressed at high night temperatures (over 26 °C). Under long days, the temperature range for tuberization was greatly restricted, with the necessity for lower night temperatures (10–17 °C). This interaction suggests that the high-temperature inhibition may operate through similar mechanism to the long-day inhibition and perhaps is subject to manipulation by the control of hormone levels [52]. Further proof of this hypothesis is provided by the increased synthesis of GA in apical buds exposed to high temperature and its transport to stolons where it inhibits tuberization [53] and that manual disbudding increased tuberization [54]. Wolf *et al.* [55] studied the partitioning of ^{14}C at 32/22, 32/12, 27/22, 27/12, and 22/12 °C day/night temperature. Neither the total plant productivity nor the export of carbon from the source leaf was affected by temperatures. More of assimilated carbon was partitioned to vegetative parts at high temperature, while at lower night temperature most of the assimilated carbon was partitioned to the tubers. They concluded that the main effect of high temperature is on assimilate partitioning and not on total plant productivity. Basu and Minhas [56] studied the partitioning of assimilated carbon within the source leaf into starch and sucrose in three heat-tolerant and three heat-susceptible varieties. They found that in heat-tolerant varieties 20–25% of the assimilates were converted to sucrose and 40–45% into starch, whereas in heat-susceptible varieties about 5% of the assimilates were converted to sucrose and 80–85% were converted to starch. Exposure of potato plants to heat stress alters the hormonal balance between roots and shoots, thus affecting tuberization and bulking. When potato plants are exposed to high temperature, gibberellin content in the leaves increases promoting haulm growth and inhibiting tuberization [53]. Basu and Minhas [57] studied gibberellins and abscisic acid concentrations in heat-tolerant and -susceptible genotypes and found that GA/ABA ratio was low in shoots of heat-tolerant genotypes and high in the shoots of heat-susceptible genotypes. GA-like substances decreased in the shoots and tended to accumulate in the roots of heat-sensitive genotypes during tuber bulking stage, whereas substantial amounts of GA-like substances remained in the shoots of heat-tolerant genotypes. They suggested that tuberization at high temperature may be related to high levels of ABA-like inhibitors in the roots during tuber induction.

45.3.3 Tuber Quality

Apart from the effects of heat stress on plant growth, development and yield, tuber quality is also affected by high temperature. Physiological disorders such as internal necrotic brown spots, chocolate spots, or internal rust spots in tuber parenchyma are linked to hot dry weather during tuber bulking [58]. Similarly, brown discoloration of the vascular ring or heat necrosis occurs at high soil temperature and varies with the severity of the stress, tuber development stage, and cultivar [59]. High temperature during harvest causes tuber rot in the irrigated soil [60]. High temperature also causes tuber shape disorders such as misshapen tubers, chain tubers, field sprouting, and reduced dry matter content [43, 61]. High temperature may cause preharvest sprouting and is linked to increased GA/ABA ratio [62].

45.3.4
Coping with Heat Stress

Potato prefers cool climate for optimum tuber yield. Therefore, the world over, the crop is grown during cool summer days in temperate zones or during short winter in subtropical zones. Most of the suitable temperature niches for potato cultivation in tropical and subtropical zones have already been occupied, leading to spatial and temporal concentration for its cultivation. To bring more area under potato, the only option available is to extend potato cultivation to less suitable areas and seasons where the crop is likely to experience high-temperature stress. Potato productivity is drastically reduced when grown under high temperatures. Therefore, development of heat-tolerant varieties is the foremost requirement for extending potato cultivation to new areas.

Genetic variability for heat tolerance exists in cultivated potato, primitive and wild species, and intergroup hybrids [63, 64]. Along with genetic variability, a good screening technique is also required for a successful breeding project. Various workers have developed screening techniques such as those based on tuberization in leaf bud cuttings, where single-node cuttings are exposed to high temperature for 3 weeks and then checked for tuberization [65] and ratio of internode length in two-node cuttings grown at high and normal temperature [66], and glasshouse screening technique for seedling populations based on their capacity to tuberize at high temperatures [67]. A combination of these screening techniques has been used by various workers for the successful development of heat-tolerant potato varieties [68, 69]. These heat-tolerant varieties have been able to extend potato cultivation to warmer nontraditional areas of Israel and India. These varieties are also a timely tool for mitigating the ill effects of global warming on potato production in the world.

Apart from heat-tolerant varieties, heat stress can also be mitigated by agronomic practices. Soil treatments like covering the soil with reflectants such as white chalk layer reduced daytime soil temperature by 7 °C, hastened emergence, foliage development, and tuber yield by more than 50% [70]. Mulch also had beneficial effects in reducing soil temperature, decreasing weed population, and increasing yield under hot dry conditions [71, 72]. Intercropping with shade crops such as maize have also shown beneficial effect; for example, shading with maize (one row of maize:three rows of potato) improved potato yield with additional maize yield coming as bonus [73, 74]. Application of calcium as fertilizer also improved the performance of potato varieties exposed to heat stress [75]. Therefore, heat stress can be successfully managed by a combination of heat-tolerant varieties and suitable agronomic practices.

45.4
Salinity Stress

Owing to an excessive use of irrigation, salinity problem in soils is increasing the world over. Moreover, vast tracts of saline soils are present in all the continents of the world. Salinity is a major cause of low crop production in many regions. Increasing

population and industrialization are taking a heavy toll on fresh water resources, and the quantity and quality of water available for agriculture are likely to decline in future. Therefore, we need to look for alternative water resources and crops that can tolerate lower quality water.

45.4.1 Ionic Imbalance in Soil

Salinity adversely affects growth and productivity of many crop plants. Soil salinity or the use of saline water results in higher osmotic potential in the soil solution, thus reducing water uptake by roots. Saline soils also have the problem of water infiltration, aeration, and root respiration [76]. Excessive concentration of certain ions such as Na, Cl, Ca, Mg, B, and SO_4 in the soil solution can cause physiological disorders in plants. In saline soils, there is an increase in adsorbed Na, a decrease in adsorbed Ca and Mg, and precipitation of Ca and Mg carbonates [77]. Saline water increases the proportion of exchangeable sodium ions in the soil solution leading to formation of sodium bicarbonate, thus raising soil pH. Resulting alkaline conditions reduce availability of nutrients such as PO_4, Fe, Mn, Zn, and so on to the plant. In calcium carbonate-rich soils, this damaging process is inhibited. This phenomenon has been exploited for saline soil reclamation by addition of gypsum. At cellular level, salinity-induced nuclear degradation in root meristematic cells was alleviated by addition of calcium to the growth medium [78].

45.4.2 Crop Growth and Yield

Affects of salinity stress are aggravated if it is combined with heat stress, water stress, high light intensity, and low humidity as encountered in natural environments [79, 80]. Crops have been rated for salt tolerance on the basis of two parameters, that is, the maximum salinity up to which there is no yield reduction and the percentage yield decrease per unit of salinity increase. According to these parameters, potato has been classified as moderately sensitive to soil salinity [81, 82]. Plant height, leaf area, and dry weight accumulation in potato are significantly reduced by salinity. Tuber yield is reduced through reduced tuber number and weight per tuber [83, 84].

Potato leaves are very sensitive to saline water and are severely damaged by sprinkler irrigation [82]. Uptake of sodium and chlorine induces toxicity as evident from leaf burn along the margins. Salinity adversely affects relative water content, stomatal conductance, and transpiration rate in potato. It also leads to changes in chloroplast ultrastructure such as thylakoid swelling and decreased grana stacking, affecting photosynthesis and reducing growth and dry matter production [85]. Irrigation with saline water during tuber germination caused greater depression in yield (59%) than when it was applied well after plant establishment (22–31%) [86]. High salinity levels (EC > 10 dS m^{-1} in the root zone) may cause coarse russetting and furrowing of tubers accompanied by severe browning of the surface, thus reducing tuber quality [87].

45.4.3
Field Selection for Salt Tolerance

Genetic variability for plant response to salinity has been found in wild potato species and potato varieties. *Solanum chacoense*, *S. kurzianum*, *S. juzepczuckii*, and *S. curtilobum* have been found to be salt tolerant in various experiments involving irrigation with saline water, glasshouse trials, and production of microtubers in the presence of NaCl in the growth medium [88, 89]. Screening for salinity tolerance has been carried out in the field by irrigation with saline water or by growing the crop in saline soils and salt-tolerant varieties such as Patrones, Norland, Red Lasoda, Cara, Serrana Alpha, Arica, and so on have been identified. These varieties fall in all maturity groups, and earliness or lateness is not related to salt tolerance [86, 90, 91]. Some of the Israeli varieties such as Almera, Hermes, and Maris Peer are not affected by moderate level of salinity, and the Peruvian variety Serrana is most salt tolerant [87].

45.4.4
Laboratory Selection for Salt Tolerance

Salt-tolerant potato lines have also been developed using recurrent *in vitro* selection of cell lines over a number of generations by exposing them to increasing salt concentration. Whole plants regenerated from salt-tolerant calli accumulated more fresh and dry weight when watered with 90 mM NaCl and also produced more tubers per plant [92]. The known salt-tolerant variety Serrana produces profuse root mass in MS medium containing 154 mM NaCl; so, plants have been screened *in vitro* by measuring root growth in culture medium containing high NaCl concentration [93, 94]. *In vitro* selection for salt tolerance has also been reported by Burgutin *et al*. [95] who identified 38 somaclones that maintained superior performance in field tests over many years.

45.4.5
Coping with Salinity

Salt-tolerant varieties have been selected using field and laboratory screening methods and are being regularly grown in many countries where good-quality water for irrigation is not available. In central Negeve desert, the underground water, too saline for irrigation, is being mixed with fresh water from the Sea of Galilee to control salinity level before applying it for irrigation to different crops [87]. This model can be easily replicated in other areas of the world facing similar problems. Sensitivity of the potato crop to salinity varies with the growth stage of the plant. Some stages are more sensitive to salinity than others [86]. Therefore, more sensitive stages can be irrigated with better quality water and the rest of the stages can be irrigated with poor-quality water to get optimum yield. In saline areas where fresh water is available, excess water can be used to leach down the salts from the top layer. In such cases, depletion of the nitrate from the root zone should be taken care of for optimum yields. Proper

fertilizer management can reduce metabolic disturbances brought about by salinity. Potassium nutrition stands out in increasing plant tolerance to salinity. It has been shown that potassium application up to 600 kg ha^{-1} increased the tuber yield of four cultivars irrigated with saline water with 9.38 dS m^{-1} electrical conductivity [96].

45.5 Reactive Oxygen Species and Abiotic Stress

Reactive oxygen species (ROS) are generated under abiotic stresses such as drought, salinity, and oxidative stress. ROS are produced in the chloroplast due to oxidative stress and is scavenged by superoxide dismutase (SOD). ROS, when produced in high amounts, cause severe damage to plant cell membranes. It has been shown that salt tolerance in potato varieties is linked to high activity of antioxidant enzymes such as peroxidase (POD) and SOD [97]. When frost-tolerant Andean potato species *S. curtilobum* and frost-sensitive *S. tuberosum* were exposed to PEG-mediated water stress, SOD activity increased by more than twofold in stress-tolerant variety and it was highly correlated with chlorophyll fluorescence parameter f_v/f_m indicating protection of PSII from ROS generated by water stress [98]. Other chemicals that scavenge ROS have also been found to enhance yield and provide protection against abiotic stresses; for example, cobalt in the culture medium provided protection to potato seedlings during osmotic stress [99]; treatment with diphenylamine, a potent antioxidant, increased potato yield by 27–47% [100]; and potato plants sprayed with antiozonant ethylenediurea (EDU) had higher amount of reduced glutathion, protected leaves against ozone damage, and increased tuber yield [101]. Treatment of plants with chlorocholine increased SOD, POD, and catalase activities, improved P, K, Ca, Mg, Fe, Mn, and Zn content, and enhanced tuber yield under suboptimal conditions [102]. Transgenic potato plants carrying bacterial catalase gene imparted salt tolerance to potato [103], proving beyond doubt the role of antioxidant enzymes in amelioration of abiotic stresses.

45.6 Conclusions

Potato produces highest amount of edible energy, protein, and dry matter per unit area and time compared to other food crops. Therefore, the crop has the potential to feed more people per unit area than any other crop. Already, more potatoes are being grown in developing countries in the tropics and subtropics than in developed countries in the temperate zones. The major limitations to potato production in tropics and subtropics are high temperatures, scarcity of water, and salinity. With increasing population and industrialization in these countries, quality and quantity of water available for agriculture are going to go down with each passing decade. These problems are likely to aggravate with impending global warming. Therefore, the study of abiotic stresses in potato crop has assumed substantial significance. It is a

challenge for scientists to use their understanding of stress tolerance mechanisms and modern technologies to develop new varieties capable of giving good yields under stressful environments. Given the genetic diversity available in potato germplasm and increased knowledge of physiology and molecular biology, the prospects are promising for our ability to improve potato yield in nontraditional environments and make more food available to millions.

References

1 van der Zaag, D.E. and Burton, W.G. (1978) Potential yield of the potato crop and its limitations. Survey papers, 7th Triennial Conference EAPR, Warsaw, Poland.
2 Minhas, J.S. and Sukumaran, N.P. (1988) *Potato Res.*, **31**, 375–378.
3 Bansal, K.C. and Nagarajan, S. (1987) *Potato Res.*, **30**, 497–506.
4 Gander, P.W. and Tanner, C.B. (1976) *Crop Sci.*, **16**, 534–538.
5 Ghosh, S.C., Asanuma, K., Kusutani, A., and Toyota, M. (2000) *Jpn. J. Trop. Agric.*, **44**, 158–166.
6 Kumar, D. and Minhas, J.S. (1994) Effect of water stress on leaf area, leaf water potential and tuber dry weight of potato cultivars Kufri Lalima and Kufri Bahar. Presented at Potato: Present & Future, Modipuram, India.
7 Walworth, J.L. and Carling, D.E. (2002) *Am. J. Potato Res.*, **79**, 387–395.
8 Basu, P.S., Sharma, A., and Sukumaran, N.P. (1998) *Photosynthetica*, **35**, 13–19.
9 Sukumaran, N.P., Ezekiel, R., and Perumal, N.K. (1989) *Photosynthetica*, **23**, 664–666.
10 Basu, P.S., Sharma, A., Garg, I.D., and Sukumaran, N.P. (1999) *Environ. Exp. Bot.*, **42**, 25–39.
11 Bezerra, F.M.L., Angelocci, L.R., and Minami, K. (1988) *Rev. Bras. Eng. Agri. Ambien.*, **2**, 119–123.
12 Minhas, J.S. and Bansal, K.C. (1991) *J. Indian Potato Assoc.*, **18**, 1–8.
13 Nooruddin, A. and Mehta, A.N. (1995) *Gujarat Agr. Univ. Res. J.*, **21**, 183–184.
14 Benam, M.B.K. and Hassanpanah, D. (2007) *Acta Hortic.*, **729**, 183–188.
15 Kumar, D. and Minhas, J.S. (1999) *J. Indian Potato Assoc.*, **26**, 7–10.
16 Susnoschi, M. and Shimsi, D. (1985) *Potato Res.*, **28**, 161–176.
17 Lugt, C., Bodleander, K.B.A., and Goodijk, G. (1964) *Eur. Potato J.*, **7**, 219–227.
18 Levy, D. (1983) *Potato Res.*, **26**, 315–321.
19 Brocic, Z., Jovanovic, Z., Stikic, R., Radovic, B.V., and Mojevic, M. (2009) *Cereal Res. Commun.*, **37** (Suppl. 1), 229–232.
20 Steckel, J.R.A. and Rgray, D. (1979) *J. Agr. Sci., Cambridge*, **92**, 375–381.
21 Deblonde, P.M.K., Haverkort, A.J., and Ledent, J.F. (1999) *Eur. J. Agron.*, **11**, 91–105.
22 Jefferies, R.A. (1993) *New Phytol.*, **123**, 491–498.
23 Balko, C. (2002) *Beitr. Zuchtun. Kultur.*, **8**, 115–118.
24 Alsharari, S.F., Alsadon, A.A., and Al-Harbi, A.R. (2007) *Acta Hortic.*, **747**, 67–74.
25 Schafleitner, R., Gutierrez, R., Espino, R., Gaudin, A., Perez, J. *et al.* (2007) *Potato Res.*, **50**, 71–85.
26 Minhas, J.S., Khurana, S.M.P., Sheshshayee, M.S., and Udaya Kumar, M. (2003) *J. Indian Potato Assoc.*, **30**, 193–194.
27 Gopal, J. and Iwama, K. (2007) *Plant Cell Rep.*, **26**, 693–700.
28 Iwama, K., Uemura, T., and Umemura, Y. (1998) *Plant Prod. Sci.*, **1**, 286–287.
29 Udaya Kumar, M., Sheshshayee, M.S., and Natraj, K.N. (1988) *Curr. Sci.*, **74**, 994–1000.

30 Steyn, J.M., Plessis, H.F.D., and Hammes, P.S. (1998) *Potato Res.*, **41**, 295–303.
31 Kawakami, J., Iwama, K., and Jitsuyama, Y. (2006) *Field Crops Res.*, **95**, 89–96.
32 Steyn, J.M., Plessis, H.F.D., Fourie, P., and Hammes, P.S. (1998) *Potato Res.*, **41**, 239–254.
33 Bhushan, B. and Sankhayan, N.K. (2002) *Res. Crops*, **3**, 584–587.
34 Wang, X.L., Li, F.M., Jia, Y., and Shi, W.Q. (2005) *Agr. Water Manage.*, **78**, 181–194.
35 Khosravifar, S., Yarnia, M., Benam, M.B.K., and Moghbeli, A.H.H. (2008) *J. Food Agric. Environ.*, **6**, 236–241.
36 Sobhani, A.R., Rahimian, H., Majidi, E., and Noormohamadi, G. (2002) *J. Agr. Sci. Islamic Azad Univ.*, **8**, 23–34.
37 Hassanpanah, D. (2009) *Res. J. Environ. Sci.*, **3**, 351–356.
38 Eiasu, B.K., Soundy, P., and Hammes, P.S. (2007) *N. Z. J. Crop Hortic. Sci.*, **35**, 25–31.
39 Belimov, A.A., Dodd, I.C., Safronova, V.I., and Davies, W.J. (2009) *Asp. Appl. Biol.*, **98**, 163–198.
40 Onder, S., Caliskan, M.E., Onder, D., and Caliskan, S. (2005) *Agr. Water Manage.*, **73**, 73–86.
41 Patel, N. and Rajput, T.B.S. (2011) *Indian J. Agr. Sci.*, **81**, 25–32.
42 Bushnell, J. (1925) The relation of temperature to growth and respiration in the potato plant. Minnesota Agricultural Experimental Station Technical Bulletin, pp. 1–29.
43 Bodleander, K.B.A. (1963) *The Growth of the Potato* (eds J.D. Ivins and F.L. Milthorpe), Butterworth., London, pp. 199–210.
44 Ewing, E.E. (1981) *Am. Potato J.*, **58**, 31–49.
45 Menzel, C.M. (1985) *Ann. Bot.*, **55**, 35–39.
46 Nagarajan, S. and Minhas, J.S. (1995) *Potato Res.*, **38**, 179–186.
47 Winker, E. (1971) *Potato Res.*, **14**, 1–18.
48 Dwelle, R.B., Kleinkopf, G.E., and Pavek, J.J. (1981) *Potato Res.*, **24**, 49–59.
49 Burton, W.G. (1981) *Am. Potato J.*, **58**, 3–14.
50 Sharma, A., Minhas, J.S., Basu, P.S., and Mohan, J. (2002) *J. Indian Potato Assoc.*, **29**, 123–127.
51 Gregory, L.E. (1953) Some factors controlling tuber formation in potato plant. Doctoral Dissertation thesis, University of California, Los Angeles.
52 Marinus, J. and Bodleander, K.B.A. (1975) *Potato Res.*, **18**, 189–204.
53 Menzel, C.M. (1983) *Ann. Bot.*, **52**, 697–702.
54 Menzel, C.M. (1981) *Ann. Bot.*, **47**, 727–733.
55 Wolf, S., Marani, A., and Rudich, J. (1990) *Ann. Bot.*, **66**, 513–520.
56 Basu, P.S. and Minhas, J.S. (1991) *J. Exp. Bot.*, **42**, 861–866.
57 Basu, P.S. and Minhas, J.S. (1999) *J. Indian Potato Assoc.*, **26**, 19–22.
58 Iritani, W.M., Weller, L.D., and Knowles, N.R. (1984) *Am. Potato J.*, **61**, 335–343.
59 Hooker, W.J. (ed.) (1981) *Compendium of Potato Diseases*, American Phytopathological Society, St. Paul, Minnesota.
60 Levy, D. (1986) *Potato Res.*, **29**, 95–107.
61 Bodleander, K.B.A., Lugt, C., and Marinus, J. (1964) *Eur. Potato J.*, **7**, 57–71.
62 Burton, W.G., van-Es, A., and Hartmans, K.J. (1992) *The Potato Crop: Scientific Basis for Improvement*, edn 2 (ed. P.M. Harris), Chapman and Hall, London, pp. 609–727.
63 Mendoza, H.A. and Estrada, R.N. (1979) *Stress Physiology in Crop Plants* (eds H. Mussell and R.C. Staples), John Wiley & Sons, Inc., pp. 227–262.
64 Levy, D., Kastenbaum, E., and Itzhak, Y. (1991) *Theor. Appl. Genet.*, **82**, 130–136.
65 Ewing, E.E. (1981) *Am. Potato J.*, **58**, 31–49.
66 Nagarajan, S. and Minhas, J.S. (1995) *Potato Res.*, **38**, 179–186.
67 Sattelmacher, B. (1983) *Potato Res.*, **26**, 133–138.
68 Levy, D., Itzhak, Y., Fogelman, E., Margalit, E., and Veilleux, R.E. (2001) *Potato Res.*, **36**, 167–173.
69 Minhas, J.S., Kumar, D., Raj, B.T., Joseph, T.A., Khurana, S.M.P. *et al.* (2006) *Potato J.*, **33**, 35–43.

70 Midmore, D. (1984) *Field Crops Res.*, **9**, 255–271.

71 Midmore, D., Berrios, D., and Roca, J. (1986) *Field Crops Res.*, **15**, 97–108.

72 Midmore, D., Roca, J., and Berrios, D. (1986) *Field Crops Res.*, **15**, 109–124.

73 Midmore, D., Roca, J., and Berrios, D. (1988) *Field Crops Res.*, **18**, 141–157.

74 Moreno, I. (1995) *Cultivo. Trop.*, **16**, 61–63.

75 Kumar, D., Minhas, J.S., and Singh, B.P. (2007) *Potato J.*, **34**, 159–164.

76 Ayers, R.S. and Westcot, D.W. (1985) *Water Quality for Agriculture*, FAO, Rome.

77 van Hoorn, J.W., Katerji, N., Hamdy, A., and Mastrorilli, M. (1993) *Agr. Water Manage.*, **23**, 247–265.

78 Richardson, K.V.A., Wetten, A.C., and Caligari, P.D.S. (2001) *Potato Res.*, **44**, 389–399.

79 Bustan, A., Sagi, M., Malach, Y.-D., and Pasternak, D. (2004) *Field Crops Res.*, **90**, 275–285.

80 Backhausen, J.E., Klein, M., Klocke, M., Jung, S., and Scheibe, R. (2005) *Plant Sci.*, **169**, 229–237.

81 Katerji, N., Hoorn, J.W., Hamdy, A., and Mastrorilli, M. (2003) *Agr. Water Manage.*, **62**, 37–66.

82 Mass, E.V. (1985) *Plant Soil*, **89**, 273–284.

83 Ghosh, S.C., Asanuma, K., Kusutani, A., and Toyota, M. (2001) *Soil Sci. Plant Nutr.*, **47**, 467–475.

84 Heuer, B. and Nadler, A. (1995) *Aust. J. Agric. Res.*, **46**, 1477–1486.

85 Fidalgo, F., Santos, A., Santos, I., and Salema, R. (2004) *Ann. Appl. Biol.*, **145**, 185–192.

86 Levy, D. (1992) *Ann. Appl. Biol.*, **120**, 547–555.

87 Levy, D. and Veilleux, R.E. (2007) *Am. Potato J.*, **84**, 487–506.

88 Bilski, J.J., Nelson, D.C., and Conlon, R.L. (1988) *Am. Potato J.*, **65**, 605–612.

89 Sabbah, S. and Tal, M. (1995) *Potato Res.*, **38**, 319–330.

90 Ahmed, R. and Abdullah, Z. (1979) *Pakistan J. Bot.*, **11**, 103–112.

91 Elkhatib, H.A., Elkhatib, E.A., Khalif-Allah, A.M., and El-Sharkawy, A.M. (2004) *J. Plant Nut.*, **27**, 1575–1583.

92 Ochatt, S.J., Marconi, P.L., Radice, S., Arnozis, P.A., and Caso, O.H. (1999) *Plant Cell Organ. Cult.*, **55**, 1–8.

93 Morpurgo, R. (1991) *Plant Breed.*, **107**, 80–82.

94 Naik, P.S. and Widholm, J.M. (1995) *Plant Cell Tissue Organ Cult.*, **33**, 273–280.

95 Burgutin, A.B., Butenko, R.G., Kaurov, B.A., and Iddagoda, N. (1996) *Russ. J. Plant Physiol.*, **43**, 524–531.

96 Elkhatib, H.A., Elkhatib, E.A., Allah, A.M.K., and El-Sharkawy, A.M. (2004) *J. Plant Nut.*, **27**, 111–122.

97 Zhang, R.J., Shang, G.B., Meng, M.L., Men, F.Y., Li, H.L., and Guo, J.X. (2007) *Chinese Potato J.*, **21**, 11–14.

98 Martinez, C.A., Loureiro, M.E., Oliva, M.A., and Maestri, M. (2001) *Plant Sci.*, **160**, 505–515.

99 Li, C.Z., Wang, D., and Wang, G.X. (2005) *Bot. Bull. Acad. Sin.*, **46**, 119–125.

100 Carrasco Rodriguez, J.L., Asensi Fabado, A., and Valle Tascon, S.D. (2005) *Water Air Soil Poll.*, **161**, 299–312.

101 Hassan, I.A. (2006) *Ann. Appl. Biol.*, **148**, 197–206.

102 Wang, H.Q., Xiao, L.T., Tong, J.H., and Liu, F.L. (2010) *Sci. Hortic.*, **125**, 521–523.

103 M'Hamdi, M., Bettaieb, T., Harbaoui, Y., Mougou, A.A., and Jardin, P.D. (2009) *Biotechnol. Agronom. Soc. Environ.*, **13**, 373–379.

46
Tobacco: A Model Plant for Understanding the Mechanism of Abiotic Stress Tolerance

Scott Schaeffer, Tyson Koepke, and Amit Dhingra

Agricultural productivity across the globe is threatened by environmental fluctuations. Years of human agronomic activity has further confounded the problem by increasing abiotic stress factors in the environment. There is an impending need for designer crops that can withstand an ever-increasing level and range of abiotic stresses to continue to support the food needs of a burgeoning population. Plants are also being explored as a source of fuel, which necessitates production of appropriate crops in nonarable lands. Thus, abiotic stress-resistant crops are relevant for both food and fuel needs in this century and beyond. Unlike biotic stresses, which are mostly monogenic traits and thus amenable to molecular breeding, abiotic stress resistance involves adjustments in morphological and physiological parameters. The multigenic nature of the trait makes it difficult, if not impossible, to breed crops in a timely manner that will be able to withstand the environmental fluctuations. This is particularly relevant for perennial crops. While annual crops can be bred in a matter of years, perennial crops stand to gain from directed biotechnological approaches. Several studies over the years in different plant species have identified various mechanisms and associated genes underlying abiotic stress resistance or tolerance. The scientific literature is replete with instances where these mechanisms were successfully unraveled in a heterologous host, tobacco that has long-served as surrogate for test of novel gene function. These studies underpin the fact that abiotic stress resistance mechanisms are shared within the plant kingdom. This chapter summarizes the current body of knowledge on our understanding of abiotic stress tolerance as deciphered from studies performed in tobacco.

46.1
Introduction

Environmental stress and in particular abiotic stress has never been more relevant to crop production than now when our planet faces climatic upheavals. Extreme temperatures resulting in freezing or drought-like conditions, extensive irrigation leading to increase in salinity and use of fertilizers for crop production over the years, and other soil quality issues related to heavy metal pollution threaten agricultural

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productivity. The situation is compounded by the need to sustain a burgeoning population estimated to rise to 9.1 billion by 2050 [1]. A recent effort to utilize crops as a source of fuel has further complicated the situation as it breeds competition for arable lands [2]. It necessitates generation of food and fuel crops suited to extreme environments to meet the world's food and fuel needs in this century and beyond.

Over eons of evolution, plants have developed tolerance mechanisms so that they can withstand environmental fluctuations. These mechanisms are wired in the genetic architecture of the plant and are called adaptation. In addition, plants can acclimate to a given stress by repeated low-grade exposure and then withstand fluctuations in abiotic conditions. This narrow window of resilience to stressful conditions is termed as phenotypic plasticity that does not require any permanent physiological or metabolic alteration [3]. These mechanisms represent available examples in nature that scientists can use to generate abiotic stress resistant crops that are adapted to harsh environments and harbor a wide range of phenotypic plasticity.

Unlike biotic stress, where defined genetic mechanisms and targets can help counter a given scourge, a plant's response to abiotic stress engages a highly complex network of physiological, genomic, proteomic, and metabolomic components. The complexity in countering environmental fluctuations, moderate or extreme, might have been a trait that was developed by the plant during evolution as a consequence of being a sessile inhabitant of the planet. It is no wonder then that genetic, molecular biology, and recently the genomics approaches have unearthed multifarious mechanisms that could be targeted for equipping desired crops to withstand a given abiotic stress. Eventual goal is to arm the plant with innate physiological mechanisms that help it in maintaining cellular homeostasis in the face of transient or chronic abiotic stress.

A large body of information exists on molecular mechanisms of abiotic stress tolerance in plants that has led to the identification of several genes or genetic loci involved in the process [4]. Since the establishment of genetic transformation strategies in the early 1980s, efforts have been underway to develop abiotic stress-resistant or -tolerant agricultural crops. Abiotic stress is a multigenic trait; that is, it is regulated by multiple genes. Thus, genetic improvement of crops via molecular breeding can prove to be an uphill, if not impossible, task. Furthermore, phenotypic characterization of abiotic stress resistance or tolerance is difficult due to the variation in stress sensitivity at different developmental stages. The observed resilience to abiotic stresses is a result of adjustments at the morphological or physiological state of the whole plant, a trait difficult to track and manipulate during breeding experiments. In contrast, resistance mechanisms are mediated by genomic, proteomic, and metabolic entities that are open for biotechnological manipulations [4]. In a rapidly changing environmental context, there is a need to develop new abiotic stress-resistant crops in a timely manner. While annual crops could be bred in a matter of years, perennial crops face the impediment of juvenility in addition to the complexity of the trait to be bred.

Some of the common abiotic stresses faced by plants are high light intensity, metal toxicities, waterlogging, extremes of cold (freezing) and heat, drought, and salt [5]. When faced with these, a plant sets into motion a cascade of molecular events to adjust the cellular homeostasis in order to reduce the damaging effect of the

immediate osmotic or oxidative stress. As summarized in Figure 46.1, the message is first perceived or sensed by receptors and transduced further via calcium and MAP kinases resulting in the activation of stress-sensitive transcription factors such as CBF/DREB. As a consequence, several stress–response mechanisms are activated that may involve detoxification, a process that scavenges dangerous reactive oxygen species (ROS) species, osmoprotection, movement of ions and water, and activation

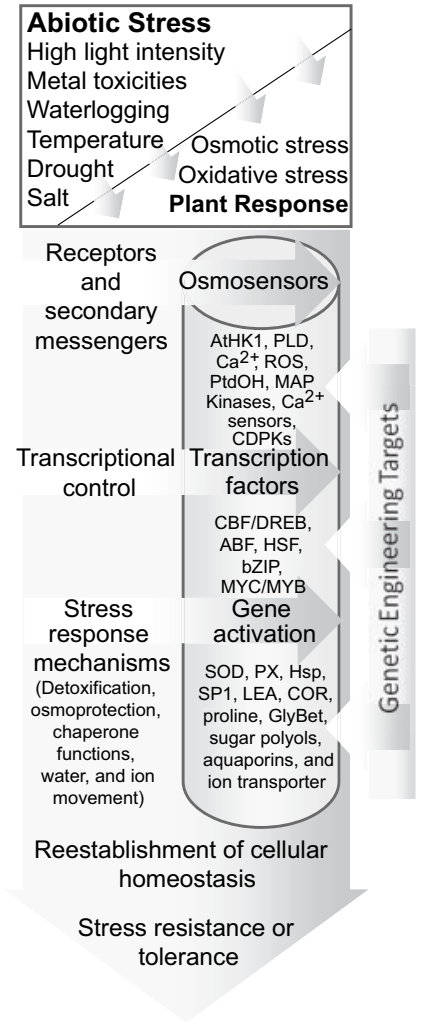

Figure 46.1 Schematic representation of the abiotic stress signal transduction pathway. Abiotic stresses trigger secondary stresses such as osmotic and oxidative stress that are sensed by receptors and transduced further via calcium and kinase cascade. Eventually activation of the involved transcriptional networks including transcription factors and associated genes engage multiple stress response mechanisms to establish cellular homeostasis. Adapted from Ref. [4].

Table 46.1 Primary affects associated with different abiotic stresses.

Primary effects	Abiotic stress
Water potential reduction	Water deficit, salinity, and freezing
Cellular dehydration	Water deficit, salinity, and freezing
Hydraulic resistance	Water deficit
Ion toxicity	Salinity
Hypoxia, anoxia	Flooding and soul compaction
Membrane destabilization	High temperature, chilling
Protein destabilization	High temperature
Symplastic ice crystal formation	Freezing
ROS production	High light intensity, trace element toxicity
Photoinhibition	High light intensity
Disturbed cofactor binding to DNA and proteins	Trace element toxicity

of chaperone function [4]. These mechanisms are facilitated by a suite of genes identified in various studies over the years.

Any imbalance in the abiotic factors manifests in a series of physiological changes termed as primary effects followed by secondary effects. Primary effects of abiotic stresses are listed in Table 46.1. Secondary effects include reduced cell/leaf expansion, reduced cellular and metabolic activities, stomatal closure, photosynthetic inhibition, leaf abscission, altered carbon partitioning, ROS production, ion cytotoxicity, cell death, fermentative metabolism, reduced CO_2 production, inadequate ATP production, and physical destruction, to name a few. Thus, any given abiotic stress never operates alone.

It is noteworthy to mention that several abiotic stress-related genes from other plant species have been successfully engineered in tobacco to obtain stress tolerance or resistance. These studies underpin the fact that abiotic stress resistance mechanisms are shared within the plant kingdom, and biotechnological manipulation may be an efficient method to ensure supply of food and fuel in the future. This chapter summarizes studies undertaken to understand the mechanism of abiotic stress resistance by testing stress-related genes in tobacco as a surrogate system that has long-served as a system of choice for testing gene function [6]. Each section is organized to provide an overview of the mechanism by which each abiotic stress condition manifests itself in a plant following which a much exhaustive list of genes is provided in a tabular format and a few relevant examples of transgenic approaches in tobacco are presented to illustrate the process.

46.2 Heat Stress

Global warming is a reality or not is a debate for another forum. Our planet is experiencing climatic change and of late extremes in weather conditions have

Table 46.2 A list of genes tested in tobacco for heat stress.

Gene	Mechanism	Gene description	References
DnaK	ROS scavenging	DnaK/HSP70	[10]
TtAPX	ROS scavenging	Ascorbate peroxidase	[11]
GST + GPX	ROS scavenging	Glutathione *S*-transferase	[12]
BADH	Osmoprotection	Betaine aldehyde dehydrogenase	[14]
AspDC	Osmoprotection	l-Aspartate-alpha-decarboxylase	[15], Fouad and Rathinasabapathi, 2006
AlSAP	Activation of stress-related genes	Stress-associated protein	[16]

become a norm. These fluctuations may not add to the average temperatures in a region but have a profound effect on field performance of plants. Short exposure, ranging from 5 min to 1 h, to high temperatures can lead to plant death.

Exposure of plants to high temperatures primarily leads to membrane and protein destabilization. The fluidity of membranes is increased resulting in leakage of ions culminating in cellular disruption. The 3D structure of individual proteins is disrupted, thereby leading to aberrant protein function and activity.

Plants experience an inhibition of photosynthesis and respiration primarily due to an imbalance between CO_2 fixed via the former process and released due to the latter. This usually happens when environmental temperature exceeds the internal temperature compensation point when the CO_2 budget is balanced by the two processes mentioned above. There is a simultaneous production of reactive oxygen species that in higher amounts triggers a cascade of events to self-destruction. Change in cellular homeostasis mediated by chronic heat stress results in cell death [7]. Several genes have been identified and tested for imparting heat stress tolerance (Table 46.2). Their molecular functions range from being molecular chaperones to providing osmotic resilience stressing the fact that multiple strategies could be utilized to engineer plants to withstand heat stress.

46.2.1
Heat Shock Proteins

As the name suggests, heat shock proteins are expressed when plants are exposed to elevated temperatures [8]. Several genomics and proteomics studies have focused on studying the role of HSPs in different plants [9]. Heat shock proteins are chaperones that facilitate protein folding and protein–protein interaction of other proteins. They are also involved in protein transport across membranes. Tolerance to heat stress has been obtained to varying degrees in different plant species by expression of HSPs. One such study in tobacco utilized a DnaK/HSP70 gene from a halotolerant cyanobacteria *Aphanothece halophytica*. Engineering of this gene in tobacco provided enhanced tolerance to heat and salt stress. The transgenic tobacco

plants exhibited higher levels of ascorbate peroxidase (APX) and catalase activity compared to the wild-type plants indicating that higher ROS scavenging may be the mechanism that provided the observed tolerance [10].

46.2.2 ROS Scavenging

Strategies to obtain heat-tolerant plants centered on ROS scavenging and detoxification abound. Generation of ROS is either a primary or a secondary effect of abiotic stress. During high-temperature stress, ROS is generated as a secondary effect and several genes have been engineered in plants to scavenge it and thereby obtain heat-tolerant plants. A chloroplast ascorbate peroxidase gene (TtAPX) from tomato was engineered in tobacco. Under high-temperature stress, there was improved seed germination, higher ascorbate peroxidase activity, and lower accumulation of hydrogen peroxide and malondialdehyde compared to the wild-type plant. In addition, the photosystem II exhibited higher photochemical efficiency [11]. In another study, ectopic expression of glutathione *S*-transferase with glutathione peroxidase activity resulted in enhanced growth of transgenic seedlings when exposed to multiple stressful conditions. Concomitant higher levels of monodehyroascorbate reductase correlated with higher glutathione and ascorbate levels. The authors concluded that the observed stress tolerance was due to glutathione-dependent peroxide scavenging resulting in reduced oxidative damage [12].

46.2.3 Osmoprotection

Heat stress disturbs the water and ion balance causing osmotic stress in the plant. Engineering efforts have centered on genes that participate in pathways producing compounds that may provide osmoprotection. One such compound is glycine-betaine, a quaternary amine widely distributed in the plant kingdom that provides plants protection against osmotic stress [13]. While there are several examples of engineering of the betaine aldehyde dehydrogenase (BADH) gene for tolerance to various stress types, in tobacco, expression of BADH resulted in accumulation of glycine-betaine [14]. The transgenic plants expressing BADH exhibited higher thermostability of the oxygen-evolving complex and the PSII reaction center. The level of H_2O_2 generated was lesser compared to wild-type plants. In another study, a prokaryotic gene L-aspartate-alpha-decarboxylase was expressed via integration in the nuclear genome. This gene catalyzes decarboxylation of L-aspartate to generate beta-alanine and CO_2. Transgenic plants had higher levels of beta-alanine, pantothenate, and total free amino acids and increased thermotolerance compared to controls [15]. In a related study, the same gene was engineered in tobacco via chloroplast transformation. It was found that PSII exhibited higher thermotolerance than in wild-type plants that may be due to osmotic protection of ROS scavenging [16].

46.2.4
Other Mechanisms

A close inspection of Figure 46.1 reveals that the protective mechanism to multiple stresses could be mediated by some common factors. A novel gene, AlSAP from *A. littoralis*, a halophytic grass, was engineered in tobacco resulting in tolerance to multiple abiotic stress conditions. The plants showed high level of resilience and successfully completed their life cycle to produce viable seeds under heat stress. The transgenic plants were also tolerant to salinity, drought, and freezing. Although the mechanism in which this gene operates is not clear, its overexpression resulted in enhancing the transcripts for eight other stress-related genes [17].

46.3
Cold Stress

Cold stress either manifests itself in the form of chilling or freezing that occurs when the temperature falls to a very low. Chilling results in membrane destabilization leading to secondary effects that are common to the heat stress. The difference is that membrane fluidity is reduced, thereby affecting several biochemical processes, ATPase activity, solute transport, energy transduction, and enzyme-dependent metabolism. Chilling injury results in vitrification and discoloration of leaves. On the other hand, freezing causes symplastic ice crystal formation. As water freezes, it expands and results in physical destruction of cellular structure [7]. Extracellular ice crystal formation may not have major damaging effect; however, intracellular ice crystal formation results in shearing of membranes and organelles. Several studies have been conducted in tobacco to understand the mechanism of cold tolerance or simply engineer the tolerance and/or resistance to cold (Table 46.3).

46.3.1
Osmotic Homeostasis

Osmotic adjustment is one of the ubiquitous mechanisms by which a plant is able to counter stress. There are several metabolic subroutines through which osmotic homeostasis can be reestablished within a plant exposed to stress. One such pathway is the chloroplast-localized glycine-betaine synthesis pathway that has been discussed elsewhere in this chapter in relation to other types of abiotic stress. Glycine-betaine provides osmoprotection to bacteria, plants, and animals. Several attempts at expressing eukaryotic genes for enzymes in glycine-betaine synthesis pathway and thus tolerance to cold proved futile. However, when a choline oxidase gene (*cod*A) from *Arthrobacter globiformis* was targeted to tobacco plastids, the resulting transgenic tobacco plants were able to withstand prolonged exposure to freezing [18, 19]. Perhaps, the *cod*A gene used in this study had better stability or was bereft of any feedback inhibition since the transgenic plants accumulated significant amount of glycine-betaine. Fructans, polymers of fructose, are also considered to play a role in

Table 46.3 List of genes engineered to confer cold tolerance/resistance in tobacco.

Gene	Gene description	References
Sod	Cu/Z superoxide dismutase	[22]
CAP 160, 85	Cold-acclimated proteins	[30]
CuCOR19	Cold-regulated gene	[125]
AtP5CS	Proline synthesis	[18, 19]
EC2.4.1.10	Levansucrase	[21]
SacB	Fructan synthesis	[18, 19]
codA	Glycine-betain synthesis	[18, 19]
OsiSAP8	Stress-associated protein	[62]
AlSAP	Stress-associated protein	[17]
ZmPP2C2	Maize protein phosphatase 2C	[126]
Bcl-xL, Ced-9	Animal cell death suppressor proteins	[32]
Glycerol-3-phosphate acyltransferase	Lipid desaturation	[25, 26]
des9	Desaturase gene	[27]
FAD7	Fatty acid desaturase	[28, 29]
JERF1	Jasmonate- and ethylene-responsive factor	[33]
CaAOC	Allene oxide cyclase	[91]
JERF3	Jasmonate and ethylene-responsive factor 3	[34]
CbLE4	Late-embryogenesis abundant	[35]
GPX	Glutathione peroxidase	[127]
GO	Glucose oxidase	[23]
tAPX	Ascorbate peroxidase	[11]
DHAR	Dehydroascorbate reductase	[94]
Nt107	Glutathione *S*-transferase	[128]

establishing osmotic homeostasis [20]. Tobacco was engineered with the constitutively expressed *Bacillus subtilis Sac*B gene coding for levansucrase, fused to the carboxypeptidase Y vacuolar sorting signal from yeast or a levensucrase gene from *Zymomona smobilis* [18, 21]. Transgenic tobacco plants with bacterial fructans demonstrated the capability to recover from freezing stress whereas wild-type tobacco plants perished [17]. The accumulation of fructans was observed to increase in the presence of cold stress. In the same study, constitutive expression of proline synthesis gene AtP5CS also resulted in tolerance to cold stress [18, 19].

46.3.2 ROS Scavenging

As shown in Figure 46.1, generation of reactive oxygen species is either a primary or a secondary effect of a given abiotic stress. There have been several instances where scavenging or prevention of ROS generation through transgene expression has been employed to attain cold tolerance. In one of the early examples, a chloroplast-targeted Cu/Zn superoxide dismutase was engineered in tobacco to confer resistance to low temperature [22]. The transgenic plants demonstrated enhanced levels of ascorbate peroxidase-specific activity suggesting enhancement of the ROS scavenging system.

More recently, direct overexpression of a thylakoid-targeted tomato tAPX (ascorbate peroxidase) gene in tobacco resulted in transgenic plants that were able to withstand low-temperature stress [11]. In another study, the concept of exogenous application of H_2O_2 in conferring abiotic stress tolerance was tested. The glucose oxidase (GO) gene from *Aspergillus niger* was expressed in tobacco. The GO expressing plants exhibited higher levels of H_2O_2 and electrolyte leakage was found to be the least in these plants when exposed to chilling temperature for 12 h [23]. The cold tolerance was perhaps associated with elevated levels of total antioxidant content and ascorbate peroxidase activity. Several other genes relating to ROS scavenging listed in Table 46.3 have also been successfully engineered to confer cold tolerance.

46.3.3
Increased Unsaturated Fatty Acids

The phosphatidylglycerol membranes of cold-resistant plants abound in unsaturated fatty acids. A desaturase enzyme enhances the amount of unsaturated fatty acids, thereby allowing the membranes to remain fluid during acclimation to cold temperatures [24]. A gene coding for glycerol-3-phosphate acyltransferase from chilling-sensitive squash was constitutively expressed in tobacco under control of the CaMV 35S promoter [25, 26]. With a similar goal, a broad-specificity Δ9 desaturase gene (des9) from the cyanobacterium *Anacystis nidulans* was engineered into tobacco [27]. The resulting transgenic plants showed reduced saturated fatty acid content in most membrane lipids and were able to survive prolonged chilling (11 days at 1 °C or 52 days at 10 °C). In another study, an *Arabidopsis* chloroplast ω-3 fatty acid desaturase gene (FAD7) was engineered in tobacco to produce transgenic plants with enhanced levels of trienoic (16 : 3 and 18 : 3) fatty acids [28]. Enhanced cold tolerance was observed in transgenic plants. The FAD7 gene was also engineered in tobacco expressed via a cold-inducible *Arabidopsis* cor15a promoter [29]. The resulting transgenic plants demonstrated greater degree of survival under prolonged chilling stress maintained at 0.5, 2.0, or 3.5 °C for 44 days, had higher levels of trienoic fatty acids, and enhanced chloroplast membrane ultrastructural stability.

46.3.4
Other Mechanisms

Cold tolerance has been imparted to transgenic tobacco by expression of cold acclimation or cold-regulated gene. Two cold acclimation proteins CAP160 and CAP85 were constitutively expressed in tobacco and the resulting transgenic plants were also crossed to obtain progeny harboring both proteins. A minor but significant cold tolerance was observed in the transgenic plants [30]. Similarly, constitutive expression of a citrus (*Citrus unshiu* Markov.) dehydrin, resulted in cold-tolerant transgenic plants [31]. The transgenic plants also exhibited more efficient germination at 15 °C compared to wild-type seeds. On the basis of *in vitro* data showing CuCOR19 protein prevented peroxidation of soybean liposomes, it is proposed that

this dehydrin may act as an ROS scavenging protein. Several other studies where animal cell death suppressor proteins, jasmonate- and ethylene-responsive factors (JERF), late-embryogenesis abundant protein, and so on have been expressed in tobacco to confer tolerance to cold [32–35].

46.4
Drought Stress

Drought stress is created due to water deficit that leads to a reduction in water potential and cellular dehydration, and creates hydraulic resistance. As a result of these primary effects of water deficit, multifarious processes malfunction. There is a reduction in cell/leaf expansion, stomatal closure, inhibition of photosynthesis, cell wall collapse called cytorrhysis, ion toxicity, leaf abscission, altered carbon partitioning, destabilization of membranes, and ultimately cell death. While drought stress is a major stress by itself, it is evident that many of the ill effects of drought are, in fact, caused by oxidative stress since some of the work targeting oxidative stress response pathways has shown to impart drought tolerance.

To examine plant responses to drought, two methods are typically used. The first, and most simple, is to reduce the amount of water given to the plants. The second involves adding an osmotic adjusting compound such as polyethylene glycol (PEG) to the water to reduce the ability of the plant to uptake water. This second method is also amenable to tissue culture work with both liquid and solid media. As mentioned previously, many of the genes conferring increased resistance to salt stress also impart drought tolerance (Table 46.4). Unfortunately, due to the nonuniformity of drought stress application and the various measurements used to examine tolerance levels, it is difficult to decipher patterns in which pathways have a larger overall effect on drought tolerance, though it is clear that there are many ways to impart drought tolerance in tobacco.

46.4.1
Osmotic Adjustment (Trehalose and Fructans)

As with the experimental method of adding compounds to water to modify the osmotic potential, producing similar compounds inside the plant can have similar effects. Trehalose, fructans, and D-ononitol have all been accumulated in transgenic tobacco to impart drought tolerance. The mechanism for these molecules to create drought stress tolerance is by reducing the water potential in the plant cells allowing them to uptake water from soil when a plant without the molecules would be unable to do so.

Trehalose 6-phosphate synthases (TPS1) perform the first step in trehalose production as shown in Figure 46.2a and have been used to produce trehalose in plant cells. Romero *et al.* [36] showed that TPS1-expressing tobacco accumulated trehalose and that increasing levels of trehalose correlated with increased drought tolerance when water was withheld for 15 days. Similarly, TPS1 showed a dose-

Table 46.4 A list of genes engineered for drought stress in tobacco.

Gene	Gene description	References
SacB	Fructan production	[129]
TPS1	Trehalose 6-phosphate synthetase	[36, 37]
IMT1	*myo*-inositol-*O*-methyl transferase	[42]
MsALR	Aldose/aldehyde reductase	[58]
AhCMO	Choline monoxygenase	[59]
ASR1	Plant-specific stress-regulated	[49]
Tsase	Trehalose synthetase	[130]
GmTP55	ALDH7 antiquitin-like proteins	[65]
P5CS	Δ1-pyrroline-5carboxylate synthetase	[131]
OsiSAP8	Stress-associated protein	[62]
BvCMO	Choline monooxygenase	[60]
AlSAP	Stress-associated protein	[17]
ApGSMT2 + ApDMT2	Methyltransferases	[61]
OsDREB1B	Dehydration-responsive element binding factor	[47]
SodERF3	Ethylene-responsive factor	[43]
JERF3	jasmonate and ethylene-responsive factor 3	[34]
PtrABF	ABA-responsive element binding factor	[48]
PsTP	Trehalose phosphorylase	[38]
GsGST	Glutathione *S*-transferase	[55]
PjGSTU1	Glutathione *S*-transferases	[56]
APX	Ascorbate peroxidase	[52]
SOD	Cu/Zn-superoxide dismutases	[50]
AtMDAR1	Monodehydroascorbate reductase	[53]
DHAR	Dehydroascorbate reductase	[132]
EC2.4.1.10	Levansucrase	[21]
GmERF3	AP2/ERF transcription factor	[44]
AnnBj1	Annexin	[63]
TPX2	Cell wall-associated peroxidase	[57]
NtHAL3a	Saccharomyces salt stress	[64]
P5CS	Δ1-pyrroline-5carboxylate synthetase	[133]
swpa4	Peroxidases	[92]
StAPX	Ascorbate peroxidase	[51]

(a) UDP-glucose + D-glucose $\rightleftharpoons$ UDP + alpha,alpha-trehalose 6-phosphate

(b) glucose and α-glucose 1-phosphate $\rightleftharpoons$ trehalose

Figure 46.2 (a) Reaction of trehalose 6-phostphate synthetase. (b) Reaction of Pleurotus sajor-caju trehalose phosphorylase and the *Grifola frondosa* Fr. trehalose synthase reaction.

dependent maintenance of photosynthetic capabilities in wild-type ($\sim$3 μmol m^{-2}), low producing transgenic ($\sim$5 μmol m^{-2}), and high producing transgenic ($\sim$10 μmol m^{-2}) lines, showing lowest to highest photosynthesis [37]. The high producing line also had increased relative water content (RWC) compared to the low producing line

and the wild type after 21 days of drought stress. Trehalose phospholylase (TP) was also used to increase trehalose production (Figure 46.2b) in tobacco. Total trehalose production reached 6.3 μmol g^{-1} tissue in the transgenic compared to ~4 μmol g^{-1} in wild type [38]. Though this increase is small, when water was withheld the wild type plants wilted by day 2 while some TP lines did not wilt even after 10 days without water. Detached leaf water retention assays demonstrated that the best TP lines lost roughly half the water the control did. Both the water withholding and detached leaf assays showed variation among the transgenic lines that suggests a possible dose-dependent effect of trehalose accumulation on drought tolerance. The third trehalose producing enzyme used by Zhang *et al.* [39] is the trehalose synthase (TSase) that produces trehalose using the same mechanism as TP. After 10 days without water followed by restarting watering, the TSase transformants had better recovery from drought stress than the wild type. Increased leaf water content, higher chlorophyll, and increased SOD and POD activities were also observed in the TSase lines.

Fructans are fructose polymers that have also been used to improve drought tolerance in tobacco. When germinated and grown on MS [40] containing 8% PEG, levansucrase (levU) expressing tobacco transformants grew the same way as unstressed, while the wild type could not grow [21]. Similarly, fructan production through another levansucrase, SacB, showed transgenic lines grew faster than wild type in 10% PEG hydroponic solution, with 19% higher fresh weight and 32% higher dry weight [41].

Production of D-(+)-ononitol from *myo*-inositol also improved drought stress tolerance [42]. For this work, a *myo*-inositol *O*-methyltransferase was expressed in tobacco and increased the total *myo*-inositol + D-(+)-ononitol to twofold the levels in the wild type. The transgenic lines showed similar reductions in photosynthesis to the wild type but recovered more rapidly upon watering after drought stress.

46.4.2 Transcription Factors

Another class of genes used to induce drought tolerance in transgenic tobacco is that of transcription factors. Owing to their DNA binding nature, transcription factors have the ability to control expression of multitudes of genes in various pathways and likely enable the crosstalk between the abiotic stresses. Several DNA binding domains are associated with abiotic stress response though the most common to date is the AP2 domain (Apetala 2).

Several AP2 containing transcription factors have been shown to improve drought tolerance when expressed in tobacco. Ethylene-responsive factors (ERFs) and JERFs are AP2 class transcription factors. When ERF3 was expressed in tobacco and the plants unwatered for 30 days, the ERF3 lines were able to grow taller than weight and flower while the wild type was not able to complete its life cycle [43]. A second set of experiments with ERF3 showed increased free proline levels and increased soluble carbohydrate accumulation [44]. These plants were then able to maintain higher root growth rates when submerged in 2% PEG compared to the control plants. When using JERF3 transgenic plants, no major differences were seen between the wild type

and the JERF3 plants during 15 days of drought; however, after 2 days of postdrought watering, all the JERF3 plants recovered, while only 20% of the wild-type plants were able to survive the 15 day drought stress [45]. Looking at JERF3 effects on other genes, it was found to upregulate many genes across the osmotic and oxidative stress pathways. CBF/DREB domains (C-repeat binding factor/dehydration-responsive element binding factor) contain an ERF/AP2 binding domain and were also tested for their ability to improve drought tolerance. Expressing DREB1A with the stress-inducible promoter rd29A yielded better growth than DREB1A with the 35S promoter under nonstressed conditions [46]. After 2 weeks without watering, both DREB1A types outperformed the wild-type plants. This work suggests that a stress-inducible promoter may enable the benefits of overexpressing stress tolerance genes without hindering plant growth under nonstress conditions. Separately, using 200 mM mannitol drought stress in solid growth media, DREB1B expressing tobacco lines had higher germination rates, overall growth chlorophyll retention, and decreased lipid peroxidation compared to the control plants [47]. The same solid growth media with 15% PEG instead of mannitol revealed higher growth inhibition for the wild type and a reduced inhibition in DREB1B plants. After 21 days without watering, DREB1B lines had up to 135% more water retention based on fresh and dry weights. As expected, the DREB1B lines did show an increase in expression of several stress-related genes.

Another transcription factor, the ABA-responsive binding factor (ABF) has also showed improved drought tolerance in tobacco [48]. The ABF lines lost less water in a dehydration assay and had lower ion leakage than the wild type. Over 3 weeks without water, ABF lines had more growth and higher amounts of chlorophyll than the wild type. Overexpression of ABF also led to upregulation of POD, SOD, and CAT (catalase).

Asr1, a zinc-dependent DNA binding peptide of unknown function, was expressed in tobacco [49]. A leaf detachment assay showed the Asr1 plants lost less water than the wild-type leaves. They also showed increased expression of 12 genes, many of which are known to be associated with stress responses.

46.4.3
ROS Scavenging Pathways

Reactive oxygen species are a major cause of damage from drought stress as verified by expression of many ROS mitigating genes improving drought tolerance. The overexpression of one of the major ROS reducing genes seen in Figure 46.3 appears to stimulate the expression of the others to some degree so the effect of each individual gene is difficult to decipher.

Superoxide is a primary reactive oxygen species that can be reduced into hydrogen peroxide by superoxide dismutase (SOD). SOD-overexpressing tobacco showed less rapid decreases in photosynthesis during drought stress compared to wild type when plants were exposed to either lack of watering or watering with 10% PEG [50].

The second step of this pathway involves ascorbate peroxidase that reduces ascorbate into monodehydroascorbate (MDA) to reduce the hydrogren peroxide

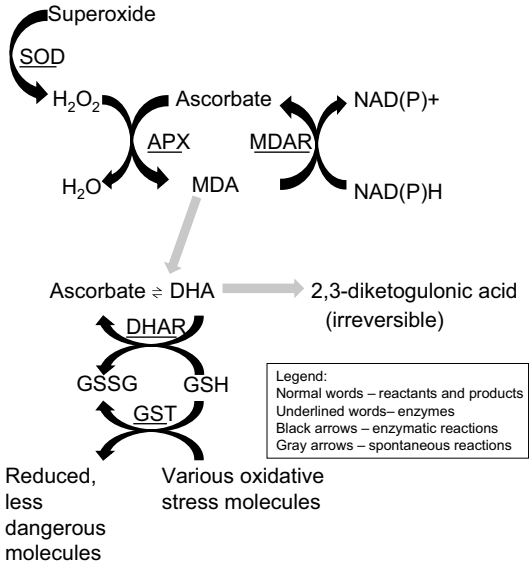

Figure 46.3 ROS generation and scavenging pathways in plants.

into water. APX-overexpressing tobacco lines showed 91–92 and 70–65% germination on 0.1 and 0.3% mannitol media, while the wild type had 70 and 24% germination [51]. This work found that hydrogen peroxide measurements were one-third to one-quarter of the wild-type levels during the stress. The authors also identified a decrease in the inhibition of PSII in the APX line. When total plant weight was measured, the APX lines showed higher growth in 300 mM mannitol, but the sample size was insufficient to find statistical significance. APX was also expressed using a glutathione reductase promoter resulting in a 3.8-fold increase in APX activity [52]. Net photosynthesis measurements showed 49.5 and 65.3% photosynthesis in transgenic and 16.2 and 58% in the wild type when comparing 10 days without water and 10% PEG treatments against normally watered control.

Once ascorbate is oxidized by APX, the MDA needs to be recycled into ascorbate or it will spontaneously disproportionate into dehydroascorbate (DHA) or ascorbate. The MDA to ascorbate reaction is carried out by MDA reductase (MDAR) and expression of MDAR has shown improved retention of starting photosynthetic rates after 8 days of 10% PEG stress [53]. Similarly, DHA reductase (DHAR) reduces DHA using glutathione (GSH) to regenerate ascorbate and if this does not occur rapidly enough, DHA can spontaneously and irreversibly degrade into 2,3-diketogulonic acid [53]. Eltayeb *et al.* [54] expressed DHAR in the cytosol and decreased the photosynthetic inhibition initiated by 8 days of either withholding water or watering with 10% PEG compared to wild type with the same treatments.

Another class of enzymes that use reduced GSH are the glutathione *S*-transferases (GSTs). These enzymes have various specificities and are involved in reducing many molecules from oxidized states. Expressing GSTs in tobacco resulted in a sixfold increase in GST activity and reduced wilting during 2 weeks without watering

compared to the wild type [55]. The GST lines also recovered from the drought stress almost immediately while the wild type did not recover and died. In tissue culture experiments, 4% mannitol hindered wild-type growth and the GST plants were phenotypically normal in up to 8% mannitol that killed all of the wild-type plants. Tissue-cultured plants on 15% PEG expressing an auxin-inducible GST from *Prosopis juliflora* (PjGSTU1), which has both GST and glutathione peroxidase (GSX) activities in *Escherichia coli*, retained turgor pressure longer than control plants [56].

Several other plant peroxidases (POD) have been used to improve drought tolerance in tobacco. Swpa4, a secratory class III peroxidase, decreased bleaching and maintained higher PSII activity following 8 days of drought stress [56]. Swpa4 plants also had an eightfold increase in H_2O_2 production leading to upregulation of many pathogen response genes. Since different levels of POD did not affect stress levels significantly, the response does not appear to be dose dependent but is rather turned on by swpa4 expression. Expression of a cell wall peroxidase (TPX2) increased the germination rate on mannitol likely due to decreased seed pore size and increased water uptake [57].

Aldose or aldehyde reductase (ALR) acts to reduce lipid peroxidation product 4-hydroxynon-2-enal. When unwatered for 35 days, ALR expressing plants were able to retain photosynthetic capabilities and recover after watering, while wild-type plants did not [58]. As expected, the transgenic lines also showed lower lipid peroxidation levels.

46.4.4 Protectants (Glycine-Betaine)

Glycine-betaine production improves overall plant health during multiple stresses including drought stress. Choline monooxygenase, CMO, is the last step in glycine-betain production and CMO transgenic plants showed 48.7% germination in 5% PEG media and 16.5% in 10% PEG media compared to 15.7 and 1.6%, respectively, for the wild type [59]. After 2 months in 10% PEG media, CMO shoot segments were still growing while the wild type had stopped growing or died. Similarly, plastid expression of CMO allowed the plants to remain unaffected after 15 days without water and the wild type completely wilted and did not recover after the end of the stress [60]. In addition, the transgenic had ~70% germination in 300 mM mannitol containing media and the wild type only had ~20% germination.

Two methyltransferases from the glycine-betain production pathway, GSMT2 and DMT2 (named T2), were expressed in tandem and produced more glycine-betain than betA lines [61]. In media with 20% PEG, T2 lines showed better germination than either betA or wild-type seeds and better than wild-type seeds in 5, 10, and 15% PEG. Through a 7 day drought period, the T2 and betA both grew more than the wild type. The relative water content and photosynthetic measurements after the 7 days of drought were highest in the T2 and lowest in the wild type. Contrarily, ion leakage and lipid peroxidation were lowest in the double transgenic and highest in the wild type. The glycine-betaine levels and their effect on drought stress demonstrate another example of dose effect of stress tolerance.

46.4.5
Other Mechanisms

While many of the genes tested in tobacco that confer drought tolerance fit into clean categories, others are not as clearly defined and are discussed in this section.

SAP8, stress-associated protein 8, showed 98 and 86% germination in 300 mM and 400 mM mannitol, respectively, while the wild type had 50 and 40% germination [62]. Overall decreases in shoot length, root length, and fresh weight were significantly higher in the wild type than in the SAP lines at each mannitol concentration as well.

Another SAP was shown to improve drought tolerance since SAP lines produced viable seeds, while wild-type plants failed to enter the reproductive phase prior to death when exposed to 25% relative soil moisture content for 30 days [17]. The role of this SAP in stress response is not yet defined.

Calcium binding annexin with many hypothesized functions was expressed in tobacco yielding transgenics that stayed green and living on 250 mM mannitol, while the wild-type tobacco became chlorotic and died [63]. During leaf disk assays in varying concentrations of mannitol, the wild type lost 70–76% of their chlorophyll and the transgenics lost only 24–34% of the chlorophyll. In addition, the wild type showed much higher rates of lipid peroxidation at all mannitol concentrations.

NtHAL3, a salt tolerance gene from *Sacchromyces cerevisiae*, yielded some lines with much higher relative growth than wild type in tissue culture media supplemented with 150 or 300 mM sorbitol after 5 days, while other lines were only slightly above wild type [64]. Plants expressing of NtHAL3 also showed increase in cellular proline.

An antiquin-like protein/aldehyde dehydrogenase, ALDH7, is involved in either general stress response or turgor pressure maintenance and its expression improved relative water content in leaves after 2 weeks without water to 52–61% from ~40% in the wild type [65].

Overall, the genes and mechanisms discussed in this section show the diversity of genes that aid in drought tolerance and provide insight into the range of genes that may be tested in the future that will have an effect on drought tolerance. Pyramiding of these genes will reveal whether the mechanisms are complementary or completely overlapping.

46.5
Salt Stress

Salt stress, specifically from sodium chloride, induces stress via several mechanisms including direct interaction of sodium ions with plant proteins and a disturbance in osmotic balance. As drought also causes stress through disruption of the osmotic balance in plant cells, factors that confer tolerance to salt stress often also confer tolerance to drought stress. These factors often relate to osmoprotectant synthesis and ROS scavenging. Damages resulting from salt-induced stresses have a severe impact on agricultural systems and are predicted to

cause the loss of up to 50% of earth's arable land by 2050 [66]. Mechanisms developed by plants to cope with living in high salt environments include salt exclusion, compartmentalization, use of osmoprotectants, and increases in protein stability [67, 68] (summarized in Table 46.5). Tobacco has been used extensively as a tool to study various mechanisms and genes involved in salt tolerance that may translate into future efforts to engineer salt tolerance or hyperaccumulation into crops (Table 46.6).

Tobacco has been engineered in numerous studies to identify the effects of the production of osmoprotectants upon increasing salt tolerance. These osmoprotectants are thought to increase stress tolerance through direct interaction and stabilization of lipids and proteins and removal of free radicals [69], in addition to the adjustment and maintenance of favorable cellular water potential [70, 71]. Overexpression of both yeast apoplastic invertase and vacuolar-targeted invertase in tobacco result in plants containing higher levels of sucrose, glucose, and fructose compared to wild-type plants in both source and sink tissues [72]. Increases in these sugars yielded plants with increased salt tolerance measured by photosynthetic activity [73].

46.5.1 Mannitol

Mannitol is a sugar alcohol that is not naturally synthesized in tobacco. Transgenic plants engineered to produce elevated levels of mannitol [74] through the overexpression of an *E. coli* gene encoding mannitol 1-phosphate dehydrogenase (*mtlD*)

Table 46.5 Salt Tolerance Osmoprotectants.

Osmoprotectant	Gene (s)	Protein	Species of origin	Reference
Mannitol	Mt1D	Mannitol 1-phosphate dehydrogenase	*Escherichia coli*	[74]
Proline	P5CS	Δ1-pyrroline-5-carboxylate synthase	*V. aconitifolia*	[77]
Glycine-betaine	*betA* *betA and betB*	choline dehydrogenase betaine aldehyde dehydrogenase	*E. coli*	[80]
D-Ononitol	*Imt1*	*myo*-Inositol *O*-methyltransferase	*M. crystallinum*	[42]
Inositol	*PINO1*	L-*myo*-inositol-1 phosphate synthase	*P. coarctata*	[63]
Trehalose	*TSase*	Trehalose synthase	*G. frondosa*	[130]
Ectoine	*Hmect. ABC*	2,4-diaminobutyrate amino transferase DABA acetyl transferase ectoine synthase	*Halomonase longata*	[135]

Table 46.6 List of genes engineered for salt tolerance in tobacco.

Gene	Gene description	Reference
Mt1D	Manitol-1phosphate dehydrogenase (mannitol synthesis)	[74]
P5cs	Pyrroline carboxylase synthetase (proline synthesis)	[77]
beta	Choline dehydrogenase (glycine-betaine synthesis)	[80]
IMT1	*myo*-Inositol-*O*-methyl transferase (D-ononitol synthesis)	[42]
Nt107	Glutatione *S*-transferase	[128]
TPX2	Cell wall-associated peroxidase	[57]
AhDnaK1	DnaK/HSP70	[68]
BjGly1	Glyoxalase 1	[96]
p5csF	Proline synthesis (feedback inhibition removed)	[78]
ApoInv	Invertase (sucrose breakdown)	[136]
PINO1	L-*myo*-inositol-1-phosphate synthase	[67]
NtHAL3a	Saccharomyces salt stress	[64]
Tsase	Trehaloses ynthetase	[130]
CHIT33 + CHIT42	Fungal endochitinase	[137]
Rab16A	Responsive to abscisic acid	[138]
swpa4	Peroxidases	[92]
DnaK	DnaK/HSP70	[10]
BADH	Betaine aldehyde dehydrogenase	[139]
BvCMO	Choline monooxygenase (plastid transformation)	[60]
PcINO1 + McIMT1	L-*myo*-inositol 1-phosphate synthase + inositol methyl transferase	[82]
betA + AtNHX1	Choline dehydrogenase + vacuolar Na^+/H^+ antiporter	[140]
AtNHX1	Vacuolar Na^+/H^+ antiporter	[83]
BvCaM	Bovine calmodulin	[141]
codA	Choline oxidase	[142]
CDH + BADH	Choline dehydrogenase + betaine aldehyde dehydrogenase	[81]
SeNHX1b + BADH	Vacuolar Na^+/H^+ antiporter + betaine synthesis gene	[143]
CMO	Choline monooxygenase	[45]
NtPI	Trypsin protease inhibitor	[144]
Bcl-xL	Animal cell death suppressor proteins	[32]
Ced-9	Animal cell death suppressor proteins	[32]
GmERF3	AP2/ERF transcription factor	[44]
GhZFP1	Zinc finger protein 1	[145]
JERF1	Jasmonate and ethylene-responsive factor	[33]
OsBIERF3	Ethylene-responsive element binding protein	[90]
JERF3	Jasmonate and ethylene-responsive factor 3	[89]
CaAOC	Allene oxide cyclase	[91]
JERF3	Jasmonate and ethylene-responsive factor 3	[34]
AhDREB1	EREBP/AP2 DNA binding protein	[146]
DAT + ASA + DABA	Ectoine biosynthetic genes	[135]
TPS	Trehalose-6- phosphate synthase	[147]

Table 46.6 (*Continued*)

Gene	Gene description	Reference
GPX	Glutathione peroxidase	[127]
GsGST	Glutathione *S*-transferase	[55]
GST + GPX	Glutathione *S*-transferase	[12]
APX	Ascorbate peroxidase – chloroplast transformation	[52]
SOD	Cu/Zn-superoxide dismutases – chloroplast transformation	[50]
OsARP	Antiporter-regulating protein	[85]
AtMDAR1	Monodehydroascorbate reductase	[53]
DHAR	Dehydroascorbate reductase	[132]
StAPX	Ascorbate peroxidase	[11]
CuZnSOD, + APX + DHAR	Superoxide dismutase + ascorbate peroxidase + dehydroascorbate reductase – chloroplast transformation	[148]
DHAR	Dehydroascorbate reductase	[94]
GmTP55	ALDH7 antiquitin-like proteins	[65]
GmNHX1	Na^+/H^+ antiporter	[84]

displayed increased salt tolerance compared to wild-type tobacco. Transgenic lines produced concentrations of 6 μmol mannitol per gram of fresh weight in the leaves and roots of some transformed plants [75]. Under prolonged exposure to 250 mM NaCl for 30 days, tobacco expressing the *mtlD* transgene experienced an 80% increase in height, while control plants grew only by 22% [74]. Mannitol accumulation promoted the growth of new leaves and roots leading to extreme differences in biomass formation between transgenic and wild-type tobacco plants. Later studies using the overexpression of *mltD* in tobacco to study salt tolerance found that overexpression of the *mltD* transgene caused a 20–25% reduction in size without application of a salt stress [76]. Under salt stress, WT plants experienced a decrease in dry weight of 44%; however, no significant decrease in dry weight occurred in tobacco overexpressing the *mltD* gene. The significant difference in growth of *mltD* overexpressing tobacco suggests that the apparent salt tolerance conferred by the overproduction of mannitol may actually be a result from the slower growth.

46.5.2 Proline

Likewise, another osmoprotectant, proline, was produced at elevated levels through transformation of a gene encoding *Vigna aconitifolia* (mothbean) Δ1-pyrroline-5-carboxylate synthase (P5CS) [77]. Plants expressing *V. aconitifolia P5CS* gene produced 10–18 times more proline than wild-type plants. Both WT and *P5CS* transgenic tobacco plants were grown to the four-leaf stage and transferred

to 400 mM NaCl solution for 3 weeks. Transgenic plants produced double the biomass and also displayed increases in flowering. Further studies looked into increasing the production of proline in tobacco via altering the feedback inhibition of proline upon P5CS [78]. *P5CS* was mutated using site-directed mutagenesis to change ala-129 to phe-129 resulting in mutant P5CS displaying low feedback inhibition [39]. Accumulation of proline in transformants overexpressing the mutated *P5CS* was double that of transformants overexpressing the wild-type P5CS gene under salt stress conditions [78]. Generation of tobacco expressing an *Arabidopsis* antisense gene encoding for proline dehydrogenase resulted in increased proline concentrations and increased salt tolerance [79]. Increased levels of proline were also induced in tobacco cells under salt stress through overexpression of the *NtHAL3* gene encoding a protein putatively involved in the coenzyme A pathway [64].

46.5.3
Glycine-Betaine

Increases in the osmoprotectant glycine-betaine were achieved in tobacco through overexpression of the *betA* gene encoding choline dehydrogenase from *E. coli* that oxidizes choline to produce betaine aldehyde [80]. Wild-type tobacco does not accumulate glycine-betaine; however, significant amounts of glycine-betaine accumulated in tobacco expressing the *betA* transgene. Expression of *betA* appeared to confer tolerance to salt stress allowing for an 80% increase in total dry weight compared to wild-type plants. Further modification of the glycine-betaine pathway was achieved through cotransformation using both *betA* and *betB*, encoding betaine aldehyde dehydrogenase [81]. Plants expressing the two transgenes accumulated two–three times more glycine-betaine than those solely overexpressing *betA* and displayed a higher rate of photosynthesis and biomass accumulation under salt stress.

46.5.4
Inositol and D-Ononitol

Tobacco plants transformed with the *IMT1* gene from the ice plant (*Mesembryanthemum crystallinum*) were able to accumulate amounts of D-ononitol exceeding 35 μmol g^{-1} fresh weight [42]. Transgenic tobacco expressing the *IMT1* gene exhibited higher photosynthetic CO_2 fixation compared to nontransgenic control plants under salt stress. Similar results were observed through engineering tobacco to express the *PINO1* gene encoding a L-*myo*-inositol-1 phosphate synthase (MIPS) from *Porteresia coarctata* (a halotolerant species of wild rice) [67]. Overexpression of the *PINO1* gene allowed for increased accumulation of inositol. Comparison of tobacco overexpressing *RINO1*, an MIPS from *Oryza sativa*, to those overexpressing *PINO1* revealed that *PINO1* transformants accumulated higher cellular inositol concentrations and maintained higher photosynthetic activities. Further studies display that overexpression of both the *IMT1* gene and the *PINO1* gene display an increased salt tolerance compared to the transformants expressing only the *IMT1* gene or the *PINO1* gene [82].

46.5.5
Sodium Transport

Sodium transport appears to be a common mechanism used by plants to control intracellular sodium concentrations. Overexpression of genes encoding antiporters in the vacuole or plasma membrane can significantly affect the salt tolerance of transgenic tobacco plants. Overexpression of *AtNHX1*, encoding a vacuolar *Arabidopsis* Na^+/H^+ antiporter, in tobacco results in plants more tolerant to salt stress [83]. Eighty-five percent of seeds from transgenic *AtNHX1* plants were able to germinate in concentrations of salt toxic to all but 5% of wild-type tobacco seeds. Activity of the vacuolar ATPase was found to be essential in maintaining the pool of cytosolic H^+ for transport to occur. In transgenic tobacco plants overexpressing *AtNHX1* vacuolar ATPase activity increased anywhere from 119 to 130% after salt stress, while wild-type plants exhibited an increase in activity of only 7%. Neither H^+-pyrophosphatase (PPase) nor plasma membrane ATPase exhibited changes in activity in either transgenic or control plants. Tolerance to salt was also conferred in tobacco through overexpression of *GhNHX1*, encoding a cotton tonoplast Na^+/H^+ antiporter [84]. Overexpression of a vacuolar antiporter regulating protein from rice, *OsARP*, conferred increased tolerance to salt stress in tobacco [85]. While the complete function of the *OsARP* product is not known, it was shown to localize to the tobacco tonoplast membrane and allow for increased storage of sodium ions in transgenic plants compared to the wild type. Through this study, OsARP is hypothesized to interact with NHX1 to increase sodium transport from the cytosol into the tonoplast.

Overexpression of either vacuolar PPase from *Thellungiella halophila* (*TsVP*) or *Arabidopsis* (*AVP1*) into tobacco conferred increased tolerance to 300 mM NaCl [86]. Vacuolar PPases function through transporting protons into the vacuole from the cytoplasm establishing a gradient from which H^+-dependent antiporters can function. At 300 mM NaCl, transgenic tobacco expressing either *AVP1* or *TsVS* accumulated 60% more dry weight than wild-type tobacco. In addition, TsVP and AVP1 transgenic lines were able to accumulate 20–30% more sodium than wild-type plants under salt stress (300 mM NaCl) conditions. Damage to the cellular membrane was reduced in both TsVP and AVP1 overexpressing tobacco, indicating that the increased ability to compartmentalize sodium ions into the vacuole significantly reduces the effects of sodium toxicity.

46.5.6
Increased Protein Stability

Increased protein stability under high saline conditions can be achieved through both the activity of stabilizing chaperone proteins and the structural modifications of proteins that lead to increased stability. DnaK/HSP70 proteins are involved in binding to proteins that have become denatured or are in nonnative states and are involved in aiding in their refolding to functional forms. Overexpression of DnaK1 from a halotolerant cyanobacterium, *A. halophytica*, in tobacco resulted in increased tolerance to high salt conditions [68].

Increased stability under high salt conditions was observed when comparing the *PINO1* gene encoding a L-*myo*-inositol-1 phosphate synthase from *Porteresia coarctata* (a halotolerant species of wild rice) to the MIPS found in *O. sativa*, *RINO1* [67]. Overexpression of *PINO1* confers the ability in tobacco to accumulate high cellular inositol concentrations under salt stress conditions, while overexpression of *RINO1* leads to lower levels of inositol accumulation. The specific activities of extracted L-*myo*-inositol-1 phosphate synthase from both *RINO1* transformants and control plants were drastically lower than that isolated from *PINO1* transformants at all tested NaCl concentrations. This difference peaked at 500 mM NaCl with almost a 10-fold increase in specific activity in *PINO1*transformant MIPS. The amino acid sequence of the MIPS from *Porteresia coarctata* and *O. sativa* differ greatly resulting in differing hydrophobicities and surface charges, suggesting such differences are important mechanisms in developing salt-tolerant proteins.

46.5.7 Exclusion

The plant cell wall is extremely important in controlling the entrance of external molecules into a plant cell. One method plants may have developed to tolerate higher levels of environmental salt is through reducing the permeability of cell and seed cell walls. Reduction of cell wall pore size was, in fact, achieved in tobacco through overexpression of *TPX2*, a gene encoding a tomato peroxidase known to play a role in modification of the cell wall structure [57]. Seeds from resulting transformants displayed a significant increase in the rate of germination (~80%) in the presence of 250 mM NaCl compared to wild-type tobacco seeds (~30%).

46.5.8 Transcription Factors: Jasmonate/Ethylene-Responsive Factors

ERFs and JERFs have been associated with promoting responses to biotic and abiotic stresses that initiate the ethylene signaling cascade [87]. These ERFs are activated by downstream participants in the ethylene cascade such as EIN 3 and EILs. ERFs are transcription factors that bind PR genes and other genes with GCC box promoters [88]. Overexpression of tomato *JERF3*, a gene whose product has high homology to only the DNA binding domain of ERF in tobacco, yielded salt tolerance [89]. Leaf disks from plants overexpressing the *JERF3* gene retained about 80% of their original chlorophyll content when placed in 300 mM NaCl, while wild-type plants maintained less than 20%. These plants expressed genes with GCC box, CE1, and DRE (dehydration responsive element)-containing promoters such as *PR* gene, as well as genes involved in ROS scavenging and photosynthetic carbon assimilation [34, 89]. A similar protein, JERF1, was produced at high levels in transgenic tobacco and displayed similar properties to JERF3; however, tobacco overexpressing *JERF1* also produced elevated levels of abscisic acid (ABA) and displayed higher vacuolar Na^+ concentrations compared to wild-type controls [33]. Salt tolerance is also conferred through the expression of rice *OsBIERF3* that was shown to induce the expression of

PR-1a [90] and soybean *GmERF3* [44]. While exact mechanisms have not been established, salt tolerance is also established in tobacco plants overexpressing a *Camptotheca acuminate* allene oxide cyclase, a key enzyme involved in the synthesis of jasmonates [91]. This study suggests that overproduction of jasmonic acid can play a significant role in developing salt tolerance; however, future studies will need to identify the molecular basis of this tolerance.

46.5.9
Reactive Oxygen Species Scavengers

As the production of reactive oxygen species is one of the major factors caused by salt stress, much attention has been given to increasing tolerance to salt stress through genetically engineering tobacco to more efficiently scavenge ROS. Engineering plants to have enhanced scavenging of reactive oxygen species has been shown to lower the toxic effects of high salt in tobacco.

Overexpression of *swpa4*, a gene encoding a peroxidase from *Imomoea batatas* (sweet potato) in tobacco, resulted in increased salt tolerance [92]. The specific activity of isolated plant peroxidases in transgenic lines expressing *swpa4* was 50 times higher compared to control plants. In addition, *swpa4* overexpressors had elevated levels of both lignin and phenolic compounds and increased chlorophyll content under salt stress conditions at both 200 and 400 mM NaCl.

Copper/zinc superoxide dismutases (Cu/Zn SOD) convert the highly reactive superoxide radical to H_2O_2. While H_2O_2 itself is cytotoxic, this reaction is the first line of defense that plants have to protect themselves against ROS. Overexpression of a gene encoding Cu/Zn SOD from *O. sativa* in tobacco chloroplasts protected against paraquat treatment and also conferred tolerance to high salt conditions [50]. Photosynthetic activity of tobacco overexpressing *Cu/Zn SOD* was significantly higher compared to the control during salt stress, and after 10 days of a recovery period maintained 79.1% of the original photosynthetic activity compared to 37.9% observed in control plants.

Ascorbate peroxidase is one of the many enzymes responsible for the scavenging of reactive oxygen species. APX catalyzes the reduction of H_2O_2 to H_2O using ascorbate as an electron acceptor that is oxidized generating a monodehydroascorbate radical (MDA). MDA can then be reduced back to ascorbate by MDA reductase or ferrodoxin. However, if this reaction does not occur in time MDA can break up disproportionately to ascorbate and dehydroascorbate. Dehydroascorbate must then be reduced through the action of dehydroascorbate reductase (DHAR), using reduced glutathione. Oxidized glutathione can then be converted back to GSH by glutathione reductase (Figure 46.3).

Overexpression of *DHAR*, *MDA*, and *APX* can significantly reduce the toxicity of salt stress-induced ROS production. Transformed tobacco overexpressing a gene encoding a thylakoid-bound APX from tomato possessed higher germination rates, photosynthetic rates, root lengths, and fresh weight and lower levels of hydrogen peroxide compared to wild-type tobacco under salt stress [51]. Tobacco chloroplast transformation using a gene encoding cytosolic APX from *Arabidopsis* also gener-

ated tobacco more tolerant to elevated levels of salt [52]. Transgenic tobacco with chloroplasts engineered to overexpress a human *DHAR*, exhibited higher levels of ascorbate and oxidized glutathionein in comparison to wild-type plants [92] and displayed increased tolerance when grown in media containing 100, 150, and 200 mMNaCl [94]. *MDA* overexpression in tobacco also conferred salt tolerance, yielding plants with higher photosynthetic rates and lower levels of hydrogen peroxide under salt stress compared to wild-type tobacco [53]. Overexpression of multiple genes in this pathway shows great promise for further increasing the efficiency of ROS scavenging under stress conditions. This was demonstrated through the overexpression of CuZnSOD, APX, and DHAR in the chloroplasts of tobacco [94]. Control tobacco, tobacco overexpressing CuZnSOD and APX (CA), and tobacco overexpressing all three genes (CAD) were grown in media containing 100 mM NaCl for 50 days. CAD tobacco had average dry shoot weights 22.3% higher than CA tobacco and 35.0% higher than control tobacco and also displayed increased root growth.

Glutathione in the reduced state is essential for the reduction of dehydroascorbate to maintain the cellular pool of ascorbate available for ROS scavenging. In addition to this role, glutathione is also utilized by the glyoxalase pathway for glutathione-based detoxification of malondialdehyde (MD) and methylglyoxal (MG), a potent cytotoxic compound produced during lipid peroxidation. Overexpression of *glyI* from *Brassica juncea* encoding glyoxylase I in tobacco conferred tolerance to MG and high salinity in transgenic tobacco [96]. Addition of *glyII* from *O. sativa* conferred greater tolerance to high methylglyoxal and NaCl concentrations [97]. Plants expressing both *glyI* and *glyII* grew under continuous salt stress and were able to flower and produce seed. Enhancing the glyoxylase pathway through tobacco genetic engineering enabled plants to resist an increase in MG levels under salinity stress, through maintenance of higher reduced:oxidized endogenous glutathione pools [98].

46.6 Metal Toxicity

Many heavy metals are necessary for plant growth and biochemical functions such as B, Co, Mn, Cu, Zn, Fe, Mg, and Mo; however, at elevated levels heavy metals can cause severe stress and toxicity in plants. These high concentrations of heavy metals are found naturally in some areas of the world as such, but are often associated with lands containing industrial pollutants. Heavy metals cause toxicity to plants via multiple mechanisms as symptoms are highly plant, metal, and dose specific. Heavy metals have been shown to promote the formation of free radicals [99, 100], compete with metal cofactors of plant enzymes, affect enzyme activity through the binding of sulphydryl and nitrogen-containing groups [101], cause cellular leakage through interactions with phospholipid head groups [99], cause disruption of ATPases, and interrupt the synthesis of lipids. Interruption or denaturation of proteins or enzymes involved in photosynthesis and respiration have been shown to generate ROS making the mitochondria and chloroplast specifically susceptible to heavy metal stress

[102, 103]. Ultimately these effects can lead to reduction in growth, chlorosis, curling of leaves, and necrosis.

Many plants have evolved mechanisms to either avoid heavy metals or prevent the interaction of these metals with cellular components. Plants that avoid heavy metals maintain the ability to either prevent the uptake of heavy metals or actively excrete these metals. Over 400 species of plants have developed methods of metal hyperaccumulation via heavy metal chelation or compartmentalization [104]. In addition to plants, many species of fungi and bacteria have developed mechanisms to overcome heavy metal stress. In order to further study the functions of these genes, tobacco has been implemented as a model system in numerous studies partly due to the ease of both nuclear and chloroplast transformation and its susceptibility to heavy metal stress. Many studies have utilized tobacco to display that certain genes are able to confer resistance to a broad range of heavy metal stresses, while others have focused on mechanisms unique to an individual heavy metal. Some of these studies are summarized in Table 46.7 and described in subsequent sections to illustrate the diversity of heavy metal tolerance mechanisms studied in tobacco.

46.6.1 Tolerance to Multiple Heavy Metals

As many heavy metals cause toxicity to plants via similar mechanisms such as ROS production, some genes have been shown to confer tolerance to stresses of multiple heavy metals.

Phytochelatins and metallothioneins are plant peptides that bind metals creating a sort of a shield that prevents or limits the interactions of metals with cellular components [105]. Phytochelatin synthase produces phytochelatins from glutathione. When present at high levels in transgenic tobacco, pytochelatin synthase transformants produce phytochelatins at elevated levels compared to wild-type plants. Transformation of tobacco with phytochelatin synthase from *Arabidopsis thaliana* resulted in the ability to accumulate cadmium within the plant at a twofold higher concentration [149]. A similar study investigated the effects of phytochelatin synthase from the cadmium hyperaccumulator *Cynodondactylon* on cadmium tolerance in tobacco [106]. Expression of this phytochelatin allowed for a 3.4-fold increase in intracellular cadmium concentrations compared to the wild-type plant. Overexpression of phytochelatin synthase from *A. thaliana* also allowed for increased tolerance to arsenic [107]. Increasing the pool of cysteine, a precursor for glutathione, through the overexpression of spinach cysteine synthase increases tolerance to cadmium, selenium, and nickel, but not lead or copper in tobacco [108]. Increases in both cysteine and glutathione were detected in cysteine synthase transformants. The increases in available glutathione likely allowed for increased phytochelatin conjugation of free heavy metals. Other phytochelatins such as the bacterial *ppk* increase mercury accumulation and tolerance in tobacco [109]. Dehydrins, another class of metal binding proteins, have also been shown to increase tolerance in tobacco to both cadmium and zinc stress through limiting ROS production leading to lipid peroxidation [110].

Table 46.7 A list of putative tolerance mechanisms associated with metal toxicity is provided.

Putative tolerance mechanism	Metal	Gene(s)	Function	Species of origin	References
Induction of ROS scavengers	Cd, Cu	*ThCHIT 33*, *ThCHIT42*	Endochitinase	*T. harzianum*	[137]
Removal of ROS cytotoxic byproducts	Cd	*MsALS*	Aldose/aldehyde reductase	*Medicago sativa*	[113, 114]
	Cd	*TvGST*	Glutathione transferase	*T. virens*	
Removal of organic groups	Hg	*merB*	Organomercury lyase	*E. coli* plasmid NR1	[122]
Overexpression of metal-containing proteins	Fe	*MsFer*	Ferritin	*M. sativa*	[119]
Volatilization	Hg	*merA*	Mercuric ion reductase	*E. coli* plasmid NR1	[122]
Metal reduction	Cd	*ArsC*	Arsenate reductase	*E. coli* plasmid R773	[112]
Chelation	Hg	*ppk*	Polyphosphate kinase	*Klebsiella aerogenes*	[109, 149]
	Cd	*AtPCS1*	Phytochelatin synthase	*A. thaliana*	[106]
	Cd	*CdPCS1*	Phytochelatin synthase	*Cynodondactylon*	[107]
	As	*AtPCS1*	Phytochelatin synthase	*A. thaliana*	[110]
	Cd, Zn	BjDHN2/BjDHN3	Dehydrin	*B. juncea*	[108]
	Cd, Se, Ni	*SpCSase*	Cysteine synthase	*Spinacia oleracea*	

Overexpression of CHIT42 and CHIT33, two fungal endochitinases yielded significantly fewer visible effects of high salt, copper, and cadmium stresses compared to wild-type plants [111]. Induction of tobacco peroxidases occurred in plants expressing both CHIT42 and CHIT33 indicating that these genes may confer heavy metal tolerance by inducing the expression of genes involved in oxidative stresses.

46.6.2 Cadmium

Compared to many other heavy metals, a significant amount of research has been performed on utilizing tobacco to study genes involved in conferring cadmium tolerance to tobacco. Overexpression of bacterial *ArsC*, encoding arsenic reductase in tobacco, yields up to a threefold increase in biomass accumulation compared to wild-type plants in media containing 100 μm $CdCl_2$ [112]. While no concrete mechanism has been associated with Cd tolerance by *ArsC*, it is hypothesized that ArsC may participate in the reduction of Cd(II) to Cd[0], a nontoxic cadmium form that may then be safely stored within the plant cell. Removal of ROS intermediates prevents the toxic effects of these ROS induced by the presence of heavy metals.

Overexpression of alfalfa/aldehyde reductase in tobacco reduces chlorosis in high concentrations of cadmium possibly through detoxification of the product 4-hydroxynon-2-enal produced via lipid oxidation [58, 113]. A similar mechanism likely confers tolerance to cadmium-induced stress in tobacco overexpressing the glutathione transferase gene from *Trichoderma virens* [114]. Glutathione transferases are thought to allow for this tolerance by binding lipid peroxidation products to glutathione.

46.6.3 Iron

Iron represents an interesting model of heavy metal stress. Plants require iron for many biological processes; however, increased levels of iron within plant cells can induce reactive oxygen species via the Fenton reaction [100, 115]. Typically, biological systems store iron reserves conjugated with proteins known as ferritins; however, excess iron is still able to cause stress [100, 116]. Transgenic studies on tobacco display that overexpression of ferritin results in lower levels of lipid peroxidation products and higher rates of photosynthesis under conditions promoting the production of ROS due to paraquat application [117]. However, overexpression of ferritins in tobacco results in phenotypes resembling iron deficiencies, as available iron pools are likely limited in the plant [118]. Overexpression of ferritins results in a 1.3–1.5-fold increase in total iron content [119].

46.6.4 Mercury

Mercury is present in many forms within the environment including Hg^{2+}, volatile Hg^0, and organic mercury, specifically methylmercury [120, 121]. Hussein *et al.* [122]

displayed that expression of *merA* and *merB* via the chloroplast genome of tobacco provided increased tolerance to mercury. *merB* encodes for a protein that removes organic groups from organomercury, which is highly toxic to plants due to interference in electron transport and chlorophyll in the chloroplasts. *merA* encodes for a protein that reduces toxic Hg^{2+} to volatile elemental Hg^{0}. With high levels of these detoxifying enzymes in the chloroplasts, transgenic tobacco was able to accumulate high mercury levels and volatize mercury at high rates compared to tobacco not expressing these genes.

An alternative approach for mercury phytoaccumulation instead of phytovolatilization was performed in tobacco [109, 123]. Nagata *et al*. engineered tobacco to express *merT*, a mercury transport protein, and *ppk*, a bacterial polyphosphate kinase that is able to chelate mercury ions and prevent volatilization. The mercury transport protein drastically increased the uptake of mercury compared to *ppk* only plants at Hg^{2+} concentrations of 2.5 μM and below. When comparing *ppk* tobacco, *ppk*/*merT* tobacco, and wild-type plants growing in media containing Hg^{2+}, both plants containing the *ppk* transgene gained the ability to accumulate significantly higher biomasses, indicating fewer effects of the toxicity of mercury. Addition of the merB gene to tobacco containing *ppk*/*merT* transgenes further increased mercury tolerance [124].

46.7 Conclusions

The convenience of being able to genetically engineer both nuclear and plastid genomes separately or simultaneously is present only in tobacco. Despite the fact that *Arabidopsis* has emerged as the dicot model over the years, tobacco continues to be employed extensively in analysis of several plant mechanisms as is evident in this chapter.

References

1 How to Feed the World in 2050, FAO (2009) (Rome).
2 Lin, L., Zhou, C.S., Vittayapadung, S., Shen, X.Q., and Dong, M.D. (2011) *Appl. Energ.*, **88**, 1020–1031.
3 Debat, V. and David, P. (2001) *Trends Ecol. Evol.*, **16**, 555–561.
4 Vinocur, B. and Altman, A. (2005) *Curr. Opin. Biotechnol.*, **16**, 123–132.
5 Mittler, R. and Blumwald, E. (2010) *Annu. Rev. Plant Biol.*, **61**, 443–462.
6 Dhingra, A., James, V.A., Koop, H.U., Mok, M.C., De Paepe, R., Gallo, M., and Folta, K.M. (2009) *Tobacco*, John Wiley & Sons, Ltd.
7 Taiz, L. and Zeiger, E. (2010) *Plant Physiology*, Sinauer Associates, Inc., Sunderland.
8 Wang, W., Vinocur, B., Shoseyov, O., and Altman, A. (2004) *Trends Plant Sci.*, **9**, 244–252.
9 Sun, W., Van Montagu, M., and Verbruggen, N. (2002) *Biochim. Biophys. Acta*, **1577**, 1–9.
10 Uchida, A., Hibino, T., Shimada, T., Saigusa, M., Takabe, T., Araki, E., and

Kajita, H. (2008) *Plant Biotechnol.*, **25**, 141–150.
11 Sun, W.H., Duan, M., Li, F., Shu, D.F., Yang, S., and Meng, Q.W. (2010) *Biol. Plant.*, **54**, 614–620.
12 Roxas, V.P., Lodhi, S.A., Garrett, D.K., Mahan, J.R., and Allen, R.D. (2000) *Plant Cell Physiol.*, **41**, 1229–1234.
13 Chen, T.H.H. and Murata, N. (2011) *Plant Cell Environ.*, **34**, 1–20.
14 Yang, X.H., Wen, X.G., Gong, H.M., Lu, Q.T., Yang, Z.P., Tang, Y.L., Liang, Z., and Lu, C.M. (2007) *Planta*, **225**, 719–733.
15 Fouad, W.M. and Rathinasabapathi, B. (2006) *Plant Mol. Biol.*, **60**, 495–505.
16 Fouad, W.M. and Altpeter, F. (2009) *Transgenic Res.*, **18**, 707–718.
17 Ben Saad, R., Zouari, N., Ben Ramdhan, W., Azaza, J., Meynard, D., Guiderdoni, E., and Hassairi, A. (2010) *Plant Mol. Biol.*, **72**, 171–190.
18 Konstantinova, T., Parvanova, D., Atanassov, A., and Djilianov, D. (2002) *Plant Sci.*, **163**, 157–164.
19 Parvanova, D., Ivanov, S., Konstantinova, T., Karanov, E., Atanassov, A., Tsvetkov, T., Alexieva, V., and Djilianov, D. (2004) *Plant Physiol. Biochem.*, **42**, 57–63.
20 Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.J., Jeuken, M.J.W., Weisbeek, P.J., and Smeekens, S.C.M. (1995) *Plant Physiol.*, **107**, 125–130.
21 Park, J.M., Kwon, S.Y., Song, K.B., Kwak, J.W., Lee, S.B., Nam, Y.W., Shin, J.S., Park, Y.I., Rhee, S.K., and Paek, K.H. (1999) *J. Microbiol. Biotechnol.*, **9**, 213–218.
22 Sengupta, A., Webb, R.P., Holaday, A.S., and Allen, R.D. (1993) *Plant Physiol.*, **103**, 1067–1073.
23 Maruthasalam, S., Liu, Y.L., Sun, C.M., Chen, P.Y., Yu, C.W., Lee, P.F., and Lin, C.H. (2010) *Plant Cell Rep.*, **29**, 1035–1048.
24 Iba, K. (2002) *Annu. Rev. Plant Biol.*, **53**, 225–245.
25 Murata, N. (1992) *Nat. Biotechnol.*, **356**, 710–713.
26 Moon, B.Y., Higashi, S., Gombos, Z., and Murata, N. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 6219–6223.
27 Ishizaki-Nishizawa, O., Fujii, T., Azuma, M., Sekiguchi, K., Murata, N., Ohtani, T., and Toguri, T. (1996) *Nat. Biotechnol.*, **14**, 1003–1006.
28 Kodama, H., Hamada, T., Horiguchi, C., Nishimura, M., and Iba, K. (1994) *Plant Physiol.*, **105**, 601–605.
29 Khodakovskaya, M., McAvoy, R., Peters, J., Wu, H., and Li, Y. (2006) *Planta*, **223**, 1090–1100.
30 Kaye, C., Neven, L., Hofig, A., Li, Q.-B., Haskell, D., and Guy, C. (1998) *Plant Physiol.*, **116**, 1367–1377.
31 Hara, M., Terashima, S., Fukaya, T., and Kuboi, T. (2003) *Planta*, **217**, 290–298.
32 Qiao, J., Mitsuhara, I., Yazaki, Y., Sakano, K., Gotoh, Y., Miura, M., and Ohashi, Y. (2002) *Plant Cell Physiol.*, **43**, 992–1005.
33 Wu, L.J., Chen, X.L., Ren, H.Y., Zhang, Z.J., Zhang, H.W., Wang, J.Y., Wang, X.C., and Huang, R.F. (2007) *Planta*, **226**, 815–825.
34 Wu, L.J., Zhang, Z.J., Zhang, H.W., Wang, X.C., and Huang, R.F. (2008) *Plant Physiol.*, **148**, 1953–1963.
35 Zhang, H., Zhou, R.X., Zhang, L.J., Wang, R.Y., and An, L.Z. (2007) *J. Plant Biol.*, **50**, 336–343.
36 Romero, C., Belles, J.M., Vaya, J.L., Serrano, R., and CulianezMacia, F.A. (1997) *Planta*, **201**, 293–297.
37 Almeida, A.M., Silva, A.B., Araujo, S.S., Cardoso, L.A., Santos, D.M., Torne, J.M., Silva, J.M., Paul, M.J., and Fevereiro, P.S. (2007) *Euphytica*, **154**, 113–126.
38 Han, S.E., Park, S.R., Kwon, H.B., Yi, B.Y., Lee, G.B., and Byun, M.O. (2005) *Plant Cell Tissue Organ Cult.*, **82**, 151–158.
39 Zhang, C.S., Lu, Q., and Verma, D.P.S. (1995) *J. Biol. Chem.*, **270**, 20491–20496.
40 Murashige, T. and Skoog, F. (1962) *Physiol. Plant.*, **15**, 473–497.
41 Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.J., Jenken, M.J.W., Weisbeek, P.J., and Smeekens, S.C.M. (1995) *Plant Physiol.*, **107**, 125–130.
42 Sheveleva, E., Chmara, W., Bohnert, H.J., and Jensen, R.G. (1997) *Plant Physiol.*, **115**, 1211–1219.
43 Trujillo, L.E., Sotolongo, M., Menendez, C., Ochogavia, M.E., Coll, Y.,

Hernandez, I., Borras-Hidalgo, O., Thomma, B.P.H.J., Vera, P., and Hernandez, L. (2008) *Plant Cell Physiol.*, **49**, 512–525.
44 Zhang, G.Y., Chen, M., Li, L.C., Xu, Z.S., Chen, X.P., Guo, J.M., and Ma, Y.Z. (2009) *J. Exp. Bot.*, **60**, 3781–3796.
45 Wu, S., Su, Q., and An, L.J. (2010) *Indian J. Biochem. Biophys.*, **47**, 298–305.
46 Kasuga, M., Miura, S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004) *Plant Cell Physiol.*, **45**, 346–350.
47 Gutha, L.R. and Reddy, A.R. (2008) *Plant Mol. Biol.*, **68**, 533–555.
48 Huang, X.S., Liu, J.H., and Chen, X.J. (2010) *BMC Plant Biol.*, **10**, 230.
49 Kalifa, Y., Perlson, E., Gilad, A., Konrad, Z., Scolnik, P.A., and Bar-Zvi, D. (2004) *Plant Cell Environ.*, **27**, 1459–1468.
50 Badawi, G.H., Yamauchi, Y., Shimada, E., Sasaki, R., Kawano, N., Tanaka, K., and Tanaka, K. (2004) *Plant Sci.*, **166**, 919–928.
51 Sun, W.H., Duan, M., Shu, D.F., Yang, S., and Meng, Q.W. (2010) *Plant Cell Rep.*, **29**, 917–926.
52 Badawi, G.H., Kawano, N., Yamauchi, Y., Shimada, E., Sasaki, R., Kubo, A., and Tanaka, K. (2004) *Physiol. Plant*, **121**, 231–238.
53 Eltayeb, A.E., Kawano, N., Badawi, G.H., Kaminaka, H., Sanekata, T., Shibahara, T., Inanaga, S., and Tanaka, K. (2007) *Planta*, **225**, 1255–1264.
54 Eltayeb, E.A., Kawano, N., Badawi, G.H., Kaminaka, H., Sanekata, T., Morishima, I., Shibahara, T., Inanaga, S., and Tanaka, K. (2006) *Physiol. Plant.*, **127**, 57–65.
55 Ji, W., Zhu, Y.M., Li, Y., Yang, L.A., Zhao, X.W., Cai, H., and Bai, X. (2010) *Biotechnol. Lett.*, **32**, 1173–1179.
56 George, S., Venkataraman, G., and Parida, A. (2010) *J. Plant Physiol.*, **167**, 311–318.
57 Amaya, I., Botella, M.A., de la Calle, M., Medina, M.I., Heredia, A., Bressan, R.A., Hasegawa, P.M., Quesada, M.A., and Valpuesta, V. (1999) *FEBS Lett.*, **457**, 80–84.
58 Oberschall, A., Deak, M., Torok, K., Sass, L., Vass, I., Kovacs, I., Feher, A., Dudits, D., and Horvath, G.V. (2000) *Plant J.*, **24**, 437–446.
59 Shen, Y.G., Du, B.X. Zhang, W.K., Zhang, J.S., Chen, S.Y. (2002) *Theor. Appl. Genet*, **105**, 815–821.
60 Zhang, J., Tan, W., Yang, X.H., and Zhang, H.X. (2008) *Plant Cell Rep.*, **27**, 1113–1124.
61 He, Y., He, C.M., Li, L.H., Liu, Z.L., Yang, A.F., and Zhang, J.R. (2011) *Mol. Biol. Rep.*, **38**, 657–666.
62 Kanneganti, V. and Gupta, A.K. (2008) *Plant Mol. Biol.*, **66**, 445–462.
63 Jami, S.K., Clark, G.B., Turlapati, S.A., Handley, C., Roux, S.J., and Kirti, P.B. (2008) *Plant Physiol. Biochem.*, **46**, 1019–1030.
64 Yonamine, I., Yoshida, K., Kido, K., Nakagawa, A., Nakayama, H., and Shinmyo, A. (2004) *J. Exp. Bot.*, **55**, 387–395.
65 Rodrigues, S.M., Andrade, M.O., Gomes, A.P.S., DaMatta, F.M., Baracat-Pereira, M.C., and Fontes, E.P.B. (2006) *J. Exp. Bot.*, **57**, 1909–1918.
66 Wang, W.X., Vinocur, B., and Altman, A. (2003) *Planta*, **218**, 1–14.
67 Majee, M., Maitra, S., Dastidar, K.G., Pattnaik, S., Chatterjee, A., Hait, N.C., Das, K.P., and Majumder, A.L. (2004) *J. Biol. Chem.*, **279**, 28539–28552.
68 Sugino, M., Hibino, T., Tanaka, Y., Nii, N., Takabe, T., and Takabe, T. (1999) *Plant Sci.*, **146**, 81–88.
69 Smirnoff, N. and Cumbes, Q.J. (1989) *Phytochemistry*, **28**, 1057–1060.
70 Galinski, E.A. (1993) *Experientia*, **49**, 487–496.
71 Bohnert, H.J. and Jensen, R.G. (1996) *Trends Biotechnol.*, **14**, 89–97.
72 Sonnewald, U., Brauer, M., von Schaewen, A., Stitt, M., and Willmitzer, L. (1991) *Plant J.*, **1**, 95–106.
73 Fukushima, E., Arata, Y., Endo, T., Sonnewald, U., and Fuihiko Sato, F. (2001) *Plant Cell Physiol.*, **42**, 245–249.
74 Tarczynski, M.C., Jensen, R.G., and Bohnert, H.J. (1993) *Science*, **259**, 508–510.
75 Tarczynski, M.C., Jensen, R.G., and Bohnert, H.J. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2600–2604.

76 Karakas, B., Ozias-Akins, P., Stushnoff, C., Suefferheld, M., and Rieger, M. (1997) *Plant Cell Environ.*, **20**, 609–616.

77 Kishor, P.B.K., Hong, Z.L., Miao, G.H., Hu, C.A.A., and Verma, D.P.S. (1995) *Plant Physiol.*, **108**, 1387–1394.

78 Hong, Z.L., Lakkineni, K., Zhang, Z.M., and Verma, D.P.S. (2000) *Plant Physiol.*, **122**, 1129–1136.

79 Kolodyazhnaya, Y.S., Titov, S.E., Kochetov, A.V., Komarova, M.L., Romanova, A.V., Koval', V.S., and Shumny, V.K. (2006) *Russ. J. Genet.*, **42**, 212–214.

80 Lilius, G., Holmberg, N., and Bulow, L. (1996) *Biotechnology*, **14**, 177–180.

81 Holmstrm, K.O., Somersalo, S., Mandal, A., Palva, T.E., and Welin, B. (2000) *J. Exp. Bot.*, **51**, 177–185.

82 Patra, B., Ray, S., Richter, A., and Majumder, A.L. (2010) *Protoplasma*, **245**, 143–152.

83 Zhou, S.F., Zhang, Z.M., Tang, Q.L., Lan, H., Li, Y.X., and Luo, P. (2011) *Biotechnol. Lett.*, **33**, 375–380.

84 Wu, C.A., Yang, G.-D., Meng, Q.-W., and Zheng, C.-C. (2004) *Plant Cell Physiol*, **45**, 600–607.

85 Uddin, M.I., Qi, Y.H., Yamada, S., Shibuya, I., Deng, X.P., Kwak, S.S., Kaminaka, H., and Tanaka, K. (2008) *Plant Cell Physiol.*, **49**, 880–890.

86 Gao, F., Gao, Q., Duan, X.G., Yue, G.D., Yang, A.F., and Zhang, J.R. (2006) *J. Exp. Bot.*, **57**, 3259–3270.

87 Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998) *Genes Dev.*, **12**, 3703–3714.

88 Zhang, H., Zhang, D., Chen, J., Yang, Y., Huang, Z., Huang, D., Wang, X.C., and Huang, R. (2004) *Plant Mol. Biol.*, **55**, 825–834.

89 Wang, H., Huang, Z.J., Chen, Q., Zhang, Z.J., Zhang, H.B., Wu, Y.M., Huang, D.F., and Huang, R.F. (2004) *Plant Mol. Biol.*, **55**, 183–192.

90 Cao, Y.F., Wu, Y.F., Zheng, Z., and Song, F.M. (2005) *Physiol. Mol. Plant Pathol.*, **67**, 202–211.

91 Pi, Y., Jiang, K.J., Cao, Y., Wang, Q., Huang, Z.S., Li, L., Hu, L.C., Li, W., Sun, X.F., and Tang, K.X. (2009) *Mol. Biotechnol.*, **41**, 115–122.

92 Kim, Y.H., Kim, C.Y., Song, W.K., Park, D.S., Kwon, S.Y., Lee, H.S., Bang, J.W., and Kwak, S.S. (2008) *Planta*, **227**, 867–881.

93 Kwon, S.Y., Ahn, Y.-O., Lee, H.-S., Kwak, S.-S. (2001) *J. Biochem. Mol. Biol.*, **34**, 316–321.

94 Kwon, S.Y., Choi, S.M., Ahn, Y.O., Lee, H.S., Lee, H.B., Park, Y.M., and Kwak, S.S. (2003) *J. Plant Physiol.*, **160**, 347–353.

95 Lee, Y.P., Kim, S.H., Bang, J.W., Lee, H.S., Kwak, S.S., and Kwon, S.Y. (2007) *Plant Cell Rep.*, **26**, 591–598.

96 Veena Reddy, V.S. and Sopory, S.K. (1999) *Plant J.*, **17**, 385–395.

97 Singla-Pareek, S.L., Reddy, M.K., and Sopory., S.K. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 14672–14677.

98 Yadav, S.K., Singla-Pareek, S.L., Reddy, M.K., and Sopory, S.K. (2005) *FEBS Lett.*, **579**, 6265–6271.

99 Hall, J.L. (2002) *J. Exp. Bot.*, **53**, 1–11.

100 Halliwell, B. and Gutteridge, J.M. (1984) *Biochem. J.*, **219**, 1–14.

101 Van Assche, F. and Clijsters, H. (1990) *Plant Cell Environ.*, **13**, 195–206.

102 Apel, K. and Hirt, H. (2004) *Annu. Rev. Plant Biol.*, **55**, 373–399.

103 Bernier, M., Popovic, R., and Carpentier, R. (1993) *FEBS Lett.*, **321**, 19–23.

104 Baker, A.J.M. and Brooks, R.R. (1989) *Biorecovery*, **1**, 81–126.

105 Cobbett, C. and Goldsbrough, P. (2002) *Annu. Rev. Plant Biol.*, **53**, 159–182.

106 Li, J.C., Guo, J.B., Xu, W.Z., and Ma, M. (2006) *J. Integr. Plant Biol.*, **48**, 928–937.

107 Li, Y.J., Dhankher, O.P., Carreira, L., Lee, D., Chen, A., Schroeder, J.I., Balish, R.S., and Meagher, R.B. (2004) *Plant Cell Physiol.*, **45**, 1787–1797.

108 Kawashima, C.G., Noji, M., Nakamura, M., Ogra, Y., Suzuki, K.T., and Saito, K. (2004) *Biotechnol. Lett.*, **26**, 153–157.

109 Nagata, T., Kiyono, M., and Pan-Hou, H. (2006) *Appl. Microbiol. Biotechnol.*, **72**, 777–782.

110 Xu, J., Zhang, Y.X., Wei, W., Han, L., Guan, Z.Q., Wang, Z., and Chai, T.Y. (2008) *Mol. Biotechnol.*, **38**, 91–98.

111 de las Mercedes Dana, M., Pintor-Toro, J.A., and Cubero, B. (2006) *Plant Physiol.*, **142**, 722–730.

112 Dhankher, O.P., Shasti, N.A., Rosen, B.P., Fuhrmann, M., and Meagher, R.B. (2003) *New Phytol.*, **159**, 431–441.

113 Hegedus, A., Erdei, S., Janda, T., Toth, E., Horvath, G., and Dudits, D. (2004) *Plant Sci.*, **166**, 1329–1333.

114 Dixit, P., Mukherjee, P.K., Ramachandran, V., and Eapen, S. (2011) *PLOS One*, **6**, 15.

115 Haber, F. and Weiss, J. (1932) *Naturwissenschaften*, **20**, 948–950.

116 Theil, E.C. (1987) *Annu. Rev. Biochem.*, **56**, 289–315.

117 Hideg, E., Torok, K., Snyrychova, I., Sandor, G., Szegedi, E., and Horvath, G.V. (2007) *Photosynth. Res.*, **91**, 308–309.

118 Van Wuytswinkel, O., Vansuyt, G., Grignon, N., Fourcroy, P., and Briat, J.F. (1999) *Plant J.*, **17**, 93–97.

119 Goto, F., Yoshihara, T., and Saiki, H. (1998) *Transgenic Res.*, **7**, 173–180.

120 Meagher, R.B. (2000) *Curr. Opin. Plant Biol.*, **3**, 153–162.

121 Patra, M. and Sharma, A. (2000) *Bot. Rev.*, **66**, 379–422.

122 Hussein, H.S., Ruiz, O.N., Terry, N., and Daniell, H. (2007) *Environ. Sci. Technol.*, **41**, 8439–8446.

123 Nagata, T., Nakamura, A., Akizawa, T., and Pan-Hou, H. (2009) *Biol. Pharm. Bull.*, **32**, 1491–1495.

124 Nagata, T., Morita, H., Akizawa, T., and Pan-Hou, H. (2010) *Appl. Microbiol. Biotechnol.*, **87**, 781–786.

125 Hara, M., Terashima, S., Fukaya, T., and Kuboi, T. (2002) *Plant Cell Physiol.*, **43**, S167–S167.

126 Hu, X.L., Liu, L.X., Xiao, B.L., Li, D.P., Xing, X., Kong, X.P., and Li, D.Q. (2010) *J. Plant Physiol.*, **167**, 1307–1315.

127 Yoshimura, K., Miyao, K., Gaber, A., Takeda, T., Kanaboshi, H., Miyasaka, H., and Shigeoka, S. (2004) *Plant J.*, **37**, 21–33.

128 Roxas, V.P., Smith, R.K., Allen, E.R., and Allen, R.D. (1997) *Nat. Biotechnol.*, **15**, 988–991.

129 Pilonsmits, E.A.H., Ebskamp, M.J.M., Paul, M.J., Jeuken, M.J.W., Weisbeek, P.J., and Smeekens, S.C.M. (1995) *Plant Physiol.*, **107**, 125–130.

130 Zhang, S.Z., Yang, B.P., Feng, C.L., and Tang, H.L. (2005) *J. Integr. Plant Biol.*, **47**, 579–587.

131 Gubis, J., Vankova, R., Cervena, V., Dragunova, M., Hudcovicova, M., Lichtnerovia, H., Dokupil, T., and Jurekova, Z. (2007) *S. Afr. J. Bot.*, **73**, 505–511.

132 Eltayeb, A.E., Kawano, N., Badawi, G.H., Kaminaka, H., Sanekata, T., Morishima, I., Shibahara, T., Inanaga, S., and Tanaka, K. (2006) *Physiol. Plant.*, **127**, 57–65.

133 Yamchi, A., Jazii, F.R., Mousavi, A., and Karkhane, A.A. (2007) *J. Plant Biochem. Biotechnol.*, **16**, 9–15.

134 Holmstrom, K.O., Somersalo, S., Mandal, A., Palva, T.E., and Welin, B. (2000) *J. Exp. Bot.*, **51**, 177–185.

135 Moghaieb, R.E.A., Tanaka, N., Saneoka, H., Murooka, Y., Ono, H., Morikawa, H., Nakamura, A., Nguyen, N.T., Suwa, R., and Fujita, K. (2006) *Plant Cell Environ.*, **29**, 173–182.

136 Fukushima, E., Arata, Y., Endo, T., Sonnewald, U., and Sato, F. (2001) *Plant Cell Physiol.*, **42**, 245–249.

137 Dana, M.D., Pintor-Toro, J.A., and Cubero, B. (2006) *Plant Physiol.*, **142**, 722–730.

138 RoyChoudhury, A., Roy, C., and Sengupta, D.N. (2007) *Plant Cell Rep.*, **26**, 1839–1859.

139 Yang, X.H., Liang, Z., Wen, X.G., and Lu, C.M. (2008) *Plant Mol. Biol.*, **66**, 73–86.

140 Duan, X.G., Song, Y.J., Yang, A.F., and Zhang, J.R. (2009) *Physiol. Plant.*, **135**, 281–295.

141 Olsson, P., Yilmaz, J.L., Sommarin, M., Persson, S., and Bulow, L. (2004) *Plant Sci.*, **166**, 1595–1604.

142 He, P.M., Zhang, D.B., Liang, W.Q., Yao, Q.H., and Zhang, R.X. (2001) *Acta Biochim. Biophys. Sin.*, **33**, 519–524.

143 Zhou, S.F., Chen, X.Y., Zhang, X.G., and Li, Y.X. (2008) *Biotechnol. Lett.*, **30**, 369–376.

144 Srinivasan, T., Kumar, K.R.R., and Kirti, P.B. (2009) *Plant Cell Physiol.*, **50**, 541–553.

145 Guo, Y.H., Yu, Y.P., Wang, D., Wu, C.A., Yang, G.D., Huang, J.G., and Zheng, C.C. (2009) *New Phytol.*, **183**, 62–75.

146 Shen, Y.G., Yan, D.Q., Zhang, W.K., Du, B.X., Zhang, J.S., Liu, Q., and Chen, S.Y. (2003) *Acta Botanica Sinica*, **45**, 82–87.

147 Jun, S.S., Yang, J.Y., Choi, H.Y., Kim, N.R., Park, M.C., and Hong, Y.N. (2005) *J. Plant Biol.*, **48**, 456–466.

148 Young-Pyo, L., Kim, S.H., Bang, J.W., Lee, H.S., Kwak, S.S., and Kwon, S.Y. (2007) *Plant Cell Rep.*, **26**, 591–598.

149 Pomponi, M., Censi, V., Di Girolamo, V., De Paolis, A., di Toppi, L.S., Aromolo, R., Costantino, P., and Cardarelli, M. (2006) *Planta*, **223**, 180–190.

Section IIID Oil Crops Including Brassicas

Improving Crop Resistance to Abiotic Stress, First Edition.
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47
Sunflower: Improving Crop Productivity and Abiotic Stress Tolerance

Carlos A. Sala, Mariano Bulos, Emiliano Altieri, and María Laura Ramos

Conventional breeding has been successful in constantly raising the sunflower (*Helianthus annuus* var. *macrocarpus*) yield potential and its stability. This improvement has been possible through both the direct manipulation of several genes controlling resistance to fungal diseases, pests, and parasitic weeds and the indirect selection of quantitative trait loci that control heritable variability of the traits and physiological mechanisms that determine biomass production and its partitioning. However, this approach may now be insufficient, since genetic progress has been slower in recent decades, and it is necessary to provide improvements at a rapid pace due to the redistribution of sunflower production toward marginal areas, due to the rapid changing cultural practices such as no-till planting or weed management, and due to the increases in the frequency and severity of abiotic constraints because of global climate change. Research in the last decades led to three main approaches to change the objectives and the current tools for sunflower breeding. First of all, plant physiology provided new tools and models to understand the complex network of yield- and stress-related traits in order to identify target traits useful to improve selection efficiency. Second, molecular genetics has led to the discovery of a large number of loci affecting yield under potential and stress conditions or the expression of stress tolerance-related traits. Third, molecular biology has provided genes that are useful either as candidate sequences to dissect QTL or for transgenic approaches. In this chapter, we reviewed and discussed molecular breeding strategies to improve sunflower yield potential and its tolerance to abiotic stresses and xenobiotics, emphasizing the requirement to face this task through an integrated multidisciplinary approach based on plant genetics and genomics, physiology, and modeling.

47.1
Introduction

Sunflower (*Helianthus annuus* L. var. *macrocarpus* Ckll.) is grown all over the world with three main purposes: beauty (ornamental sunflower), direct consumption of the seeds (confectionary sunflower), and oil production (oilseed sunflower). By far,

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the last of them is the most important objective in terms of acreage and production [1] and is the subject of this chapter. Sunflower oil has been traditionally viewed as a healthy vegetable oil and it is considered a premium oil for salad, cooking, and margarine production [2] and is also being evaluated as a source of biodiesel [3].

With a cultivated acreage of over 22 million ha and an annual production of around 9 million ton, sunflower is grown on every continent, but its production is mainly concentrated in the Russian Federation, Ukraine, India, and Argentina. Sunflower oil is the fourth most important vegetable oil in world trade after soy, palm, and canola oils. Unlike soybean, sunflower is primarily an oil crop, with high protein meal being a by-product. The world production of sunflower pellets is also important, as it is the principal grinding subproduct. Argentina is the leading exporter and the European Union is the greatest importing block [4].

Morphological, geographical, molecular, and archeological evidence indicates that sunflower domestication took place in eastern North America [5–7], where it was used as a source of food, pigment, and medicine by the Native American Indians [8]. A substantial genetic bottleneck occurred during domestication, both at the nuclear [9] and at the plasmon levels [7]. In fact, the cultivated sunflower gene pool has retained only 40–50% of the nucleotide diversity that can be found in wild sunflower populations [10].

The transformation of sunflower into a major oilseed crop, however, took place only in the second half of the twentieth century due to two major breeding achievements: the drastic increase in oil percentage in sunflower achenes achieved in the former Soviet Union from 1920 to 1960 [11] and the development of a cytoplasmic male sterility system [12] combined with fertility restoration by nuclear genes [13] that enabled the commercial production of hybrid seed [14, 15]. Even though domestication and breeding create population bottlenecks and eroded genetic diversity in sunflower [6, 9, 10], diverse and complex parentage and migration have apparently partially counteracted the effects of domestication and other diversity-reducing processes in modern oilseed sunflower inbred lines [16]. Significant nucleotide diversity was discovered across inbred lines despite the effects of genetic drift and the winnowing of unfavorable alleles through intense selection and inbreeding in single-cross hybrid sunflower breeding programs. Surprisingly, nucleotide diversity was estimated to be 1.7-fold greater in elite inbred lines than primitive and early open-pollinated (OP) cultivars. In fact, nucleotide diversity in sunflower is only slightly lower than maize, two- to fivefold greater than other domesticated grasses, eight- to tenfold greater than soybean, and several-fold greater than other autogamous plant species [17].

Crop performance is the end result of the action of thousands of genes and their interactions with environmental conditions and cultural practices. Conventional breeding has been very successful in constantly raising the sunflower yield potential and its stability. This improvement has been possible through the direct manipulation of several genes controlling resistance to fungal diseases, pests, and parasitic weeds, and the indirect selection of quantitative trait loci (QTL) that control heritable

variability in the traits and physiological mechanisms that determine biomass production and its partitioning. This last approach came into being with little or no knowledge of the factors governing the genetic variability exploited by breeders for crop improvement. However, this approach may now be insufficient since it is necessary to provide improvements at a rapid pace due to the redistribution of sunflower production toward marginal areas and due to the increase in the frequency and severity of abiotic constraints because of global climate change. Cold stress, drought, and salinity will probably become more prevalent in certain areas, while there will be an increased demand for agricultural products and reduced availability of agricultural land and natural resources such as water and fertilizers. Finally, breeding also needs to exploit positive interactions with rapid changing cultural practices such as no-till planting or weed management.

We review and discuss published results about molecular breeding strategies to improve sunflower yield potential and its tolerance to abiotic stresses and xenobiotics, emphasizing the requirement to face this task through a multidisciplinary approach based on plant genetics and genomics, physiology, and modeling.

47.2 Breeding Achievements

Scientific sunflower breeding was started in 1910–1912 at Krasnodar by Vasilii Stepanovich Pustovoit, an academic, based on the varieties locally developed during the nineteenth century [18]. The efforts of breeders were initially devoted mainly to genetically control parasitic weeds (broomrape, *Orobanche cumana*) and insects (sunflower moth, *Homeosoma electellum*), but the development of varieties with high oil content by Pustovoit became a milestone in the evolution of sunflower as an oil crop throughout the world. The local varieties cultivated in Russia in 1913 contained only 30–33% of oil in dry seeds. This percentage increased up to 43% in 1935, 46% in 1953, and 51% in 1958, when the variety "Peredovik" was released [18]. This spectacular increase in oil content of the achenes did not cause any decline in the seed yield of the varieties released. The open pollinated Russian cultivar Peredovik, with high oil content, introduced during the 1960s in the Western countries (the United States, Canada, Western Europe, and Argentina), was the basis of the first sustained commercial production of oilseed sunflower in these countries [19].

The discovery of cytoplasmic male sterility, with its inherent advantages, provided a highly efficient method for commercial production of hybrid seed and was the second milestone in the development of sunflower. The first stable source of cytoplasmic male sterility was discovered by Patrice Leclercq in 1968 from an interspecific cross involving *H. petiolaris* and *H. annuus* [12]. Subsequent identification of genes for fertility restoration in wild species [13] and in certain obsolete sunflower cultivars [20] allowed an efficient and economical production of hybrid seed. The development of the first sunflower hybrids based on cytoplasmic male

sterility in the early 1970s intensified the interest of seed companies in the crop, which led to a considerable increase in sunflower production in many countries. When comparing sunflower yields in the countries that grew open-pollinated varieties before the introduction of hybrids, seed yields increases of about 20% were estimated [19].

Information regarding breeding achievements with respect to seed and oil yields after the initial introduction of hybrids is scarce. The main exceptions are the results reported for Argentina [21–25]. Taking into account that approximately 1.9 million ha of sunflower is grown in Argentina between latitudes 26° S (Chaco province) and 39° S (southern Buenos Aires province), that this area includes a wide range of environmental conditions (subtropical and temperate climates and different types of soils) and management practices, breeding achievements for sunflower in Argentina are a representative example of other regions in the world and will be considered in this chapter.

The relative contributions of plant breeding and crop management to yield improvement over time in a given cropping region can be separated (e.g., [26, 27]). Genetic gains can be estimated by comparing a historic set of cultivars with uniform management practice or the trial data collected by breeding programs. This gain in relative terms is subtracted from the total gain in farmers' fields and the residual is assumed to be due to changes in management practices [28].

Using this approach and a set of sunflower cultivars released in Argentina between 1930 and 1995, López Pereira *et al.* [21] found that both grain and oil yields were positively associated with the year of cultivar release and that there was a clear discontinuity in yield trends with a marked step around 1970, when the first hybrids were released. On average, hybrids outyielded open-pollinated cultivars by 23% for grain and 36% for oil. No improvement in yield potential, however, was apparent during long periods before and after this turning point (Figure 47.1). These authors hypothesized that the historic requirement for disease tolerance and grain quality, together with a rather narrow genetic base, has imposed restrictions on the improvement in yield potential. In further studies, focusing on the genetic gain by selection in the period between 1983 and 1998, Sadras *et al.* [24] found a positive association between oil yield and year of commercial release, which was related to both resistance to fungal diseases (specially verticillium wilt, caused by *Verticillium dahliae*, [29, 30]) and response to intraspecific competition.

Using meta-analysis of multienvironment trials [31], de la Vega *et al.* [32] quantified increases in oil yield and determined the contributions of change in both biotic stress resistance and yielding ability in favorable environments for sunflower hybrid varieties released during the period 1995–2005. Genetic gains came about due to both an increase in the number of hybrids with resistance to the major biotic stress (*V. dahliae*) and a genetic gain in oil yield of 14.4 kg ha^{-1} yr^{-1} in these resistant hybrids. It is likely that at least part of the slowdown observed in grain yield gains in the national data during 1995–2005 was a result of a breeding process that, for that period, increased oil yield mostly through an increase in grain oil concentration [32] and, also, because of the presence of large and regional genotype × environment (G × E) interactions [25].

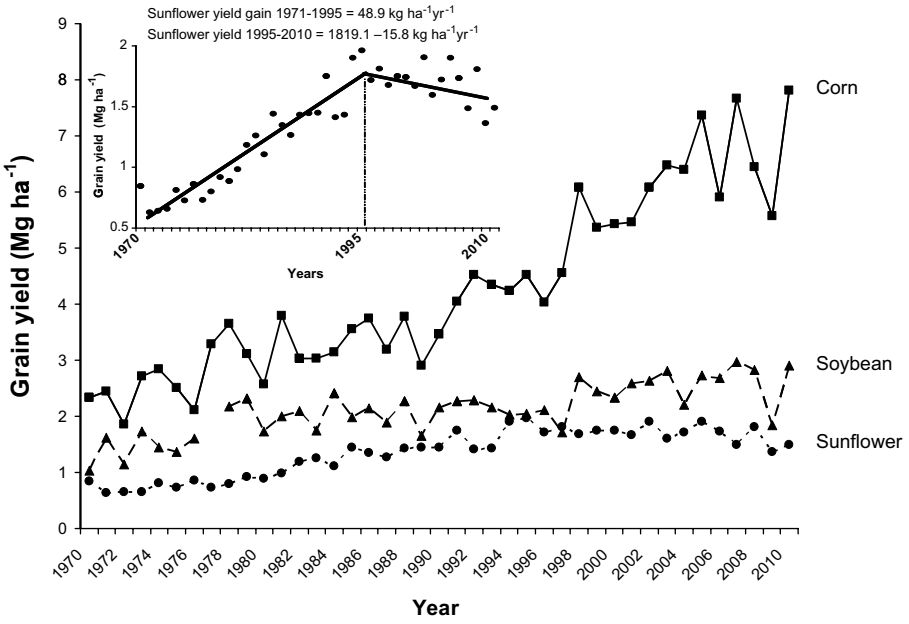

Figure 47.1 Comparison of sunflower, maize, and soybean grain yields per hectare in Argentina over the past 40 years. Two main aspects permit to explain the bilineal relationship between mean sunflower yields and year, as shown in the box: (a) national data accounts only for mean grain yield and not oil content that was a main breeding objective during the past two decades; (b) the explosive growth of soybean in Argentina, which increased from 6.0 million ha planted area in 1994 to more than 15 million ha in 2010, has pushed sunflower production to more marginal areas [32] (data obtained from [298]).

47.3
Identification of Key Traits Useful for Increasing Yield Potential and Abiotic Stress Tolerance

Conceptually, the environmental effect on a genotype depends on three main elements: soil, cultural practices, and weather. Soil and cultural practices are usually persistent from year to year and can be regarded as fixed. The weather element is more complex because it has a persistent part represented by the general climatic zone and an unpredictable part represented by time variation (year to year). Once the environmental effect has been conceptually subdivided into predictable and unpredictable components, a similar subdivision can be made for the G×E interaction [33, 34]. Understanding of the underlying physiology of the genotype-specific responses to predictable and unpredictable environmental variation would improve the efficiency of selection within a complex target population of environ-

ments [25, 35, 36]. On the other hand, it permits to identify key traits as targets for selection to cope with the predictable and unpredictable elements of the environment in a given region, namely, (i) attributes that can be selected to achieve adaptation to the target environment by emphasizing predictable interactions (e.g., phenology and photoperiodic response; duration of grain filling, a trait associated with canopy stay green; and salt and cold tolerance) and (ii) attributes that allow the unpredictable G × E interactions to be accommodated, improving yield stability in a target production environment (e.g., osmotic adjustment, leaf expansion, etc.).

Sunflower is a glycophyte species moderately tolerant to salinity and is considered in the same tolerance category as soybean [37]; therefore, it can be grown successfully on most agricultural soils [38]. However, if the competence with other crops requires the expansion of the sunflower crop to regions with saline soils, apparently there exists enough genotypic variability and medium-to-high heritabilities for salt tolerance in sunflower, in order to develop salt-tolerant sunflower hybrids by conventional breeding methods [39–41]. In addition, genetic resources from wild species [42–46] have been exploited to develop salt-tolerant lines [47, 48].

Higher yield stability in regions with marked interyear variability in rainfall, a characteristic of drought-prone areas, involves the identification, testing, and breeding for particular attributes. Identification of such attributes can be assessed by comparing a range of genotypes over several years, under both irrigated and rainfed conditions that produced terminal droughts. This approach was followed by Fereres *et al*. [49] and Giménez and Fereres [50] in an extensive comparative analysis of drought tolerance in sunflower. They showed that yield under drought was closely associated with harvest index and found intraspecific variability in root depth, which in turn was linked to cultivar maturity type. They also identified variability for both sensitivity of leaf conductance to leaf water potential and for osmotic adjustment (OA). Furthermore, they found no association between yield potential and susceptibility to drought indicating that the development of high-yield, drought-tolerant cultivar is possible [51].

As was pointed out by Connor and Hall [51], evidence suggests that it should be possible to breed for tolerance to some categories of stress in sunflower without loss of yield potential because there are firm indications of intraspecific variability in traits that confer tolerance (see Ref. [52] for a review). The likelihood of success will be increased if work is geared toward the identification of key attributes based on a good understanding of the causal relationships between the presence of a trait and the physiology of yield loss minimization under stress [53]. Further progress will depend on the introduction in high-yield genotypes of traits able to improve stress tolerance without detrimental effects on yield potential, thus reducing the gap between yield potential and yield in stress-prone environments. This goal can be achieved through the identification of stress tolerance-related traits and the subsequent manipulation of the corresponding genes using marker-assisted selection (MAS) and/or gene transformation.

47.4
Linkage Mapping

47.4.1
Genetic Linkage Maps and other Genomic Resources

Over the past decades, several genetic linkage maps differing in length and density were developed for cultivated sunflower (a paleopolyploid, [54]; with $2n = 2x = 17$ chromosomes) or for crosses between cultivated and wild sunflowers (see Refs [15, 55] for reviews). These maps are based on different molecular markers such as restriction fragment length polymorphism (RFLP) and/or random amplification of polymorphic DNA (RAPD) markers for the first reported maps [56–63]. RAPDs have been used primarily for tagging phenotypic loci in sunflower, for example, rust (*Puccinia helianthi* Schw.) and broomrape-resistance genes [64, 65]. Later on, the addition of Amplified Fragment Lenght Polymporphism (AFLPs) [66–68] and direct amplification of length polymorphism markers (DALPs) [69] allowed further saturation of genetic linkage maps. The distribution of DALPs and AFLPs revealed that both markers tagged different regions to enable covering most of the sunflower genome.

Another multipoint marker developed for sunflower and used for mapping purposes are the so-called target region amplification polymorphisms (TRAPs), using EST database information to generate polymorphic markers around targeted candidate gene sequences [70]. The TRAP technique has been employed in sunflower to construct a linkage map [71], to define the sunflower linkage group (LG) ends through the use of TRAP markers based on *Arabidopsis*-type telomere repeat sequences [72], to map several traits (e.g., *ms9*, [73]; a gene for downy mildew resistance, [74]; the chlorophyll-deficient mutation *yl*, [75]; the fertility factor *Rf1*, [76]), and to assess germplasm relationships.

Two of the RFLP maps have been used as tools for mapping phenotypic and quantitative trait loci [77–85]; however, the widespread use of RFLP markers and maps in sunflower has been restricted by lack of public RFLP probes, a consequent lack of a dense public RFLP map, and low-throughput nature of RFLP markers. The difficulties posed by the historic lack of public, single-copy DNA markers were only slightly offset by the emergence of facile, universal DNA markers, such as RAPDs, AFLPs, and TRAPs. While RAPD, AFLP, and TRAP markers have a multitude of uses, they are dominant, multicopy, and often nonspecific in nature and, as a whole, unsatisfactory for establishing a genome-wide framework of DNA markers for anchoring and cross-referencing genetic linkage maps. Single-copy, codominant DNA markers are preferred for such purposes and until 2002 were lacking in sunflower [86].

The concomitant development of a large number of simple sequence repeat (SSR) markers and the automation of mapping procedures [87–89] eliminated the long-standing bottleneck caused by the scarcity of single-copy DNA markers in the public domain and supplied the critical mass of DNA markers needed to create a public reference map, unify independently developed molecular genetic linkage maps, and establish an universal LG nomenclature. Tang *et al.* [88] constructed the first genetic

linkage map for sunflower on the basis of SSR markers and the first dense public genetic linkage map on the basis of single- or low-copy DNA markers. Since the three RFLP maps of sunflower lacked common, public domain DNA markers, QTL and other trait mapping results could not be universally exploited, compared, or validated, this resource created the basis for rapidly, efficiently, and fully integrating first-generation genetic linkage maps developed by using RFLP markers. Yu *et al.* [90] integrated and cross-referenced the Tang *et al.* [88] SSR map with the RFLP maps of Berry *et al.* [58] and Jan *et al.* [59] using the Gedil *et al.* [67] RFLP map as a bridge. Insertion–deletion (INDEL) markers were also developed from RFLP markers by sequencing the cDNA clones, aligning sunflower cDNA and *Arabidopsis* genomic DNA sequences, predicting from such an alignment intron sites in sunflower genes, and designing flanking primers to amplify the introns and flanking coding regions spanned by the primer pairs. The density and utility of the molecular genetic linkage map of cultivated sunflower was increased by adding unmapped SSR markers developed by European researchers (Cartisol, CRTx SSRs), together with those already developed by North American (ORSx SSR markers) and South American researchers (INTA Argentina, Hax markers; [87]). These efforts contributed to the availability of more than 2000 SSR markers for mapping purposes in sunflower [55]. All the reported linkage maps can be easily viewed and compared using a useful web-based application: CMap [91].

Most of the SSR markers used in sunflower mapping are neutral (usually located in intergenic genomic regions), as they were developed from genomic libraries using microsatellite motives as hybridization probes [87–89]. In recent years, due to the rapid increase in sequence information, sunflower transcript assemblies were built and mined to identify SSRs and INDELs for marker development, comparative mapping, and other genomics applications in sunflower. In fact, more than 320 000 expressed sequence tags (ESTs) have been generated and are available for sunflower and related species of the genus *Helianthus* [92]. The information can be accessed through the Compositae DataBase [93], the GenBank dbEST division [92], the Compositae Genome Project [94], and the Gene index accessible through the GenBank UniGene division [95].

To create a transcript map for sunflower, Lai *et al.* [96] identified 605 ESTs that displayed small INDEL or single-nucleotide polymorphism (SNP) variations *in silico*, had apparent tissue-specific expression patterns, and/or were ESTs with candidate functions in traits such as development, cell transport, metabolism, plant defense, and tolerance to abiotic stress. Primer pairs for 535 of the loci were designed from the ESTs and screened for polymorphism in recombinant inbred lines (RIL). In total, 273 of the loci amplified polymorphic products, of which 243 mapped to the 17 LGs of sunflower. Comparisons with previously mapped QTL revealed some cases where ESTs with putatively related functions mapped near QTLs identified in other crosses for salt tolerance and for domestication traits such as stem diameter, shattering, flowering time, and achene size. The generation of EST-SSR and SNP markers complemented existing SSR marker collections [97, 98] allowing the inclusion of functional markers in genetic maps [96, 99]. Through direct sequencing of sunflower genomic regions, belonging to a small group of inbreds and landraces, more than

1700 SNPs and 147 INDELs were obtained [10, 17, 100]. Recently, a restriction site-associated DNA sequencing technology for SNP discovery identified more than 1400 unique contigs with at least one high-quality SNP [101].

Sequencing more diverse EST libraries within cultivated sunflower or from wild sunflower species could enhance the number of polymorphisms discovered. It is questionable, however, whether all these polymorphisms would also be segregating in the available mapping populations. As shown in maize and its wild relative teosinte [102], cultivated maize harbors much less polymorphism than teosinte. A framework to integrate all the reported information concerning sunflower genomics and to generate the basis for future research is the Sunflower Genome Sequencing Project [103], which is still in progress.

47.4.2
Recombinant Inbred Lines Used for Mapping Purposes

Populations of RILs have been used for developing reference linkage maps of the sunflower genome and for QTL mapping studies. Two of them were used and reported on several occasions and for this reason both of them will be described here in some detail.

The population of RILs from the cross RHA266 × PAC2 was developed by the INRA in France. RHA266 is a restorer, unbranched, oilseed sunflower line, released in 1971 by the USDA-ARS and Texas Agricultural Experiment Station [104]. It is based on Peredovik germplasm crossed by a line with rust resistance, 953-102-1-1-41. PAC2 is a line developed by INRA and was obtained from a cross between the USDA line HA61 and a wild *H. petiolaris* population [57].

The other population of RILs was developed by US researchers from the cross RHA280 × RHA801 [88]. RHA280 is a restorer, unbranched, nonoilseed sunflower (confectionary) inbred line developed by selection from Sundak and released in 1974 by the USDA. RHA801 is a restorer, branched, oilseed sunflower line, developed and released by USDA in 1981, and derived from a restorer population with a complex pedigree after one cycle of recurrent selection for improved yield [105].

These populations represent interesting tools for mapping studies because they were used as a framework for developing linkage maps obtained with different kinds of molecular markers. However, their use as populations to identify yield or stress-related traits may be limited by the little differences between their parental lines for many of the features under study and because none of them is a high-yield modern inbred line, so the QTL obtained using these RILs may now be ubiquitous in the elite germplasm.

47.4.3
QTL Mapping

Yield and tolerance to abiotic stress are complex traits regulated by a number of factors that can be studied as component traits. The development of different

genetic maps for sunflower allowed genetic dissection of the quantitative traits controlling a wide range of physiological characteristics related to oil yield and the adaptive response of sunflower to abiotic stress. This is a prerequisite to allow cost-effective applications of genomics-based approaches to breeding programs aimed at improving the sustainability and stability of yield under adverse conditions.

47.4.3.1 Oil Yield

Most cultivated sunflower is grown as a source of vegetable oil. Thus, the principal goal of sunflower breeding programs is to develop F_1 hybrid cultivars with high oil yield. Sunflower oil yield per unit area is determined by the product of seed yield per unit area and oil percentage in the seed. Therefore, consideration of both components is important when breeding for high oil yield hybrids.

Seed oil concentration is a complex trait determined by the genotype and the environmental conditions. Search for seed oil concentration QTL using a genetic map of 205 loci defined by RFLP [81] and composite interval mapping resulted in the detection of eight QTL on seven LGs that accounted for 88% of the phenotypic variation for seed oil concentration across environments [79]. Gene action was additive for four QTL and dominant or overdominant for the others. Four of the eight QTL were detected in two or more environments, and the parental effects were the same across generations and environments. In another study, six QTL for percentage of oil in the grain were detected using a set of 244 F_3 families derived from another cross and a genetic map based on 170 AFLP and SSR markers. The percentage of phenotypic variance explained was 90.4% [106]. On 220 F_2 and 180 F_3 progenies of different genetic origin, and using a genetic map based on RFLP and SSR [107], four QTL were identified in both F_2 plants and F_3 families on four LG, which explained 68–70% of the phenotypic variance. Tang *et al.* [108] identified six QTL for seed oil content in a low- $\times$ high-oil (RHA280 $\times$ RHA801) RIL mapping population segregating for apical branching (*B*), phytomelanin pigment (*P*), and hypodermal pigment (*Hyp*) loci. The seed oil concentrations of RHA280 and RHA801 differed dramatically, from 254 to 481 g kg^{-1}, respectively. Interestingly, three of the QTL were tightly linked to *B*, *P*, and *Hyp*. The same relationship between apical branching and seed oil content was observed in other mapping populations [109, 110].

Hajduch *et al.* [111] using a proteomic approach reported 77 protein spots differentially expressed in the high oil line RHA801 versus the low oil line RHA280. Identification of 44 of these proteins indicated that the two main processes affecting low or high oil concentration in these lines were glycolysis and amino acid metabolism suggesting that seed oil content is tightly linked to carbohydrate metabolism and protein synthesis in a complex manner. Although the number of differences found by these authors should not be related only to seed oil content as they stated, since RHA801 and RHA280 are not isolines but members of rather different gene pools, their results describe the proteomic differences between confectionary and oilseed varieties.

47.4.3.2 Seed Weight

Seed weight is an important property under complex genetic and environmental control, and associated with morphological and developmental characteristics such as plant height or flowering dates. A set of 244 F_3 families was screened with AFLP and microsatellite markers and a linkage map was constructed based on 170 markers. One putative QTL for 1000-grain weight was detected explaining 5.4% of total phenotypic variance [106]. Al-Chaarani *et al.* [68] reported three QTL for this trait and one of them was a major QTL that explained 37% of the phenotypic variance for this trait.

Using a genetic map with 290 markers for a cross between two inbred sunflower lines and 2 years of observations on F_3 families, two QTL controlling seed weight were detected. Phenotypic variation explained by both QTL were 16.0 and 25.2 for F_2 an F_3 populations, respectively. Some of the QTL controlling seed weight overlapped with those controlling oil content. The QTL on the same LG as the branching gene *b1*, also reported by Mestries *et al.* [109] for another cross, was almost certainly linked to capitulum size. The second QTL for seed weight was close to the one for flowering date. The seed weight character measured is not "yield" in the agricultural sense of seed production per hectare of hybrid genotypes. It was seed weight per capitulum on selfed plants, with little involvement of heterosis [110].

In the RIL population from the cross RHA280 × RHA801 mentioned before, five QTL controlling seed weight were reported explaining 72.8% of the phenotypic variation. However, the contribution of the pleiotropic effect of the apical branching gene *B* accounted again for the bulk (52.5%) of this variation.

47.4.3.3 Days to Flowering

Sunflower can be grown under a wide variety of climatic conditions so a wide range of total crop durations are required around the world. In addition, knowledge of the relative lengths of the period from sowing to flowering, when potential seed number is determined, and of the period from flowering to maturity, when seed filling occurs, can be important in breeding for yield. Present-day sunflower varieties show wide variation in these characters.

Diversity of the production area together with the characteristic of the original germplasm base and the subsequent introduction of foreign germplasm determined the coexistence of a great variability in types of hybrids grown in Argentina. Historically, two major hybrid types of different genetic origin were grown in the Argentine sunflower production area from the 1970s to early 1990s: (i) intermediate-late to late maturity hybrids of white-striped seed, low grain oil concentration, and high relative grain yield, mostly developed from locally bred, open-pollinated varieties, and (ii) intermediate-early to early maturity hybrids of black seed, high grain oil concentration, and low relative grain yield, largely derived from Eastern European and US germplasm. Breeding and selection by recombining both types converged to a third hybrid type that combined high grain yield and high grain oil concentration with an intermediate maturity. The breeding process involved the selection of the maturities that tended to maximize yield potential and stability in each of the three megaenvironments or subregions of sunflower production in

Argentina [25]. This process reduced the original range of phenological responses within subregions, but tended to concentrate on different maturity types among megaenvironments. The modern high-yield hybrids are late, intermediate, or early compared to the mean of the old ones in the northern, central, and southern subregions of Argentina, respectively, which in turn reflects the positive, orthogonal, and negative associations between oil yield and maturity in the three subregions [25].

León *et al*. [78] mapped QTL associated with growing degree days (GDD) to flowering and photoperiod (PP) response in an elite sunflower population derived from a cross of two divergent lines representative of the two original germplasm pools: HA89 and ZENB8 [25]. HA89 exhibits an ambiphotoperiodic response (short- or long-day response depending on photoperiod); it shows its longest relative maturity when the photoperiod at emergence is about 11–13 h and a long-day response at longer day lengths. Relative to ZENB8, HA89 line types require more growing degree days to flower when the photoperiod during emergence is equal to or less than 14 h. This type of response reflects that of group II hybrids, which are relatively late in the northern subregion, where emergence and vegetative period occur under short photoperiods, and relatively early in the central and southern subregions, where later planting and higher latitudes are associated with longer photoperiods for the same crop phase. The line ZENB8 exhibits a day-neutral to short-day response, and takes more days to flower than HA89 under photoperiods longer than 14 h; this type of response being representative of that of group I hybrids.

Two hundred and thirty-five F_2-generation plants and their $F_{2:3}$ and $F_{2:4}$ progenies of a single-cross population derived from the cross HA89 × ZENB8 were evaluated in six environments (locations, years, and sowing dates) with photoperiods known to elicit a PP response between the inbred lines. Detection of QTL was facilitated with a genetic linkage map of 205 RFLP loci and composite interval mapping. Six QTL in LGs A, B, F, I, J, and L were associated with GDD to flowering and accounted for 76% of the genotypic variation in the mean environment; however, QTL in LG A and B accounted for 72% of the genetic variation and were highly dependent on PP. QTL mapping of the ratio of the GDD required by a progeny to flower at a PP of 12.1 and 15.0 h, defined as the photoperiod response (PPR), suggested that alleles at QTL in LG A and B were responsive to PP. QTL in LG F and J showed QTL × E interaction but the LOD values were not associated with PP. QTL × E interactions for additive effects were highly significant for LG A, B, and F. QTL × E interactions for QTL with dominant effects were significant for LG A, B, and J. The dominant effect of QTL in LG B increased in environments with a longer PP [78].

Given these two QTL (A and B) that are strongly associated with the photoperiod response that controls GDD to flowering, Fonts *et al*. [112] examined the phenology of near-isogenic families bearing all combinations of alleles for both of them associated with photoperiodic response when growing in controlled environment chambers under short and extended photoperiods. Plants were harvested at intervals, the apices dissected out, and apex development from the start (apex transformation) to the end of floral differentiation scored. Near-isogenic lines (NILs) exhibited significant effects of photoperiod, QTL, QTL × photoperiod, and QTL × QTL interactions for the timing of apex transformation and for the inverse of rate of development during

floral differentiation. The strong QTL A × QTL B interaction for both traits reflects a much greater delay in development under both photoperiods when QTL A was derived from HA89 and QTL B from ZENB8. Also, they found a three-factor (QTL A × QTL B × photoperiod) interaction, acting on the rate of development during the floral differentiation process [112]. Interestingly, some of the combinations of alleles at both QTL should be representatives of the type III group of high-yield hybrids grown in Argentina. These and other alleles may be used to achieve a better match between the edaphoclimatic supply and the physiological requirements during critical periods of the crop in different production areas of the world. This better adaptation to the predictable environmental variation should be complemented with a better understanding of the physiological and genetic causes underlying genotypic differences for the duration of the grain filling period.

47.4.4
Mapping QTL for Yield-Related Traits under Stress Conditions

Generally, QTL studies in sunflower have been performed under only one water regime. Such studies do not lead to separation of constitutive QTL from adaptive ones. Sorting out constitutive from adaptive QTL effects is possible by evaluation of the same mapping population under different conditions. These studies permit to investigate the genetic basis of trait association by looking for colocation of corresponding QTLs for yield-related traits on the genetic map under stress and nonstress conditions as exemplified below.

Using a set of sunflower RILs derived from the cross PAC2 × RHA266, Ebrahimi *et al.* [113] determined the effects of water stress on several seed quality traits including oil content in the seed, and mapped QTL controlling these traits under two different water treatments. Interestingly, in spite of that there were no significant differences for oil content in the seed between both parents in the four environmental conditions, significant variation among RILs was observed. Genotype × environment interaction was detected only for the RILs under greenhouse but not under field conditions. Eighteen QTL for oil content were detected under well-watered conditions and eleven under stress conditions, but only three of them were common to both treatments, although the phenotypic correlation for oil content under both water regimes was significant. Both parental lines contributed to the expression of oil content. The most important QTL for oil content was found on LG 16 and explained 16% of the phenotypic variance. The positive alleles for this QTL come from RHA266. Four other QTL for this trait under both water treatments were found on LG 16, so this region appears to be important for oil content under water-stressed and well-watered conditions. Two QTL, in LG1 and LG16, were also reported by Tang *et al.* [108] for oil content in sunflower recombinant inbred lines.

QTL controlling four chlorophyll fluorescence parameters were analyzed in the same population of RILs under well-watered and water-stressed conditions in 45 day-old plants at stage near flower bud formation. A large genetic variation and transgressive segregation were observed for the traits studied under two water

treatments. Results showed that the progressive water stress did not cause long-term downregulation of photosynthesis apparatus, but it reduced actual efficiency of PSII electron transport. QTL analysis showed that several putative genomic regions were involved in the total variation of chlorophyll fluorescence parameters under two water treatments. Among the 26 QTL detected under well-watered conditions, 5 were shown to be constitutive by QTL-by-water treatment (environment) interaction. Most of the QTL were specific for one condition, demonstrating that the genetic control of the expression of the traits related to photosynthesis differed under different water conditions. In several cases, one QTL was found to be associated with more than one trait. The results also showed overlapping QTL for some of the chlorophyll fluorescence parameters and plant water status traits described above, mainly on LGs 7 and 16 [114].

The same population of RILs was used to study agronomical traits under greenhouse and field conditions, each with two water treatments. The difference among RILs was significant for all the traits studied under all conditions; and water treatment × RIL interaction was also observed for most of the traits under both field and greenhouse conditions. Several QTL were identified for yield-related traits with the percentage of phenotypic variance explained by QTL (R^2) ranging from 4 to 40%. QTL for grain yield per plant under four water treatments were identified on different LGs, among which two were specific to a single treatment. Three QTL for grain yield per plant were overlapped with several QTL for some of the drought-adaptive traits described before [115].

Differential display analysis was used to compare overall differences in gene expression between drought- or salinity-stressed and unstressed (control) plants of sunflower. Five drought-regulated cDNAs and twelve salinity-regulated cDNAs were cloned and sequenced. Thirteen of these cDNAs were confirmed to be expressed differentially in response to drought or salinity stress by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Regulation of the expression of these 13 genes was analyzed in leaves of drought-stressed plants and in roots and shoots of drought- and salinity-stressed seedlings. Results showed that certain genes respond to both stresses, while others are uniquely regulated either in terms of the stress stimulus or in terms of the plant tissue [116]. In this context, results of QTL analysis for different traits under stress and nonstress conditions confirm the differential display results and highlight the existence of adaptive QTL (or genes) that are detected (expressed) only under specific environmental conditions or modify its expression with the level of an environmental factor, and QTL (genes) consistently detected (expressed) across most environments.

Selecting which QTL/traits follow with MAS is crucial. The improvement of drought tolerance should not be achieved with a parallel limitation of yield potential. Hence, drought-tolerance traits should be tested in both stressed and nonstressed environments before being introduced in an MAS breeding program. QTL for drought-related traits coincident with QTL for yield potential should be considered as priority targets for MAS. However, confirmation and validation of the reported QTL in different genetic backgrounds should be performed prior to their utilization in breeding.

47.4.5
Mapping QTL for Stress-Related Traits

Genetic mapping with dense marker maps can be used to identify the number and genetic positions of QTL associated with specific traits that confer yield advantages under stress conditions. In addition, this process can be used both to estimate effects of the segregating QTL and their contributions to trait variation (individually and in combined QTL models) and to obtain estimates of their stability across environments and across genetic backgrounds. Trait-based physiological approach has been used successfully in other crops to improve performance in drought-prone environments [117] and it can be used in sunflower, integrated with an MAS approach, to improve yield and yield stability efficiently.

Three target traits in sunflower that can confer yield advantages in stress-prone environments and for which QTL analyses were reported are osmotic adjustment, leaf expansion, and cold tolerance during initial stages of development, and will be reviewed below.

47.4.5.1 Osmotic Adjustment

Osmotic adjustment refers to the lowering of osmotic potential due to the net accumulation of solutes in response to water deficits [118]. Since OA helps to maintain higher leaf relative water content (RWC) at low leaf water potential (LWP), it is evident that OA helps to sustain turgor maintenance and hence growth, while the plant is meeting transpirational demand by reducing its LWP [119, 120].

Chimenti and Hall [121] examined intraspecific variation in osmotic adjustment in sunflower using a collection of 33 genotypes of different origin, which were exposed to water stress at the 8-leaf stage. Changes in osmotic adjustment with ontogeny were also evaluated in the pre- and postanthesis phases using seven genotypes drawn from this collection. Estimates of OA were derived from measurements of leaf RWC and osmotic potential during a period in which the soil was allowed to dry gradually. All genotypes at the 8-leaf stage showed some degree of OA and significant differences in RWC were found between extreme genotypes in all three ontogenetic phases. The value of OA as a trait that can contribute effectively to yield maintenance under drought in sunflower was later examined by Chimenti *et al.* [122]. They screened a set of 25 inbred lines reputed to differ for drought tolerance for OA expressed in the 8-leaf stage. They crossed the extreme lines (high and low OA) and selected four individuals (two high OA, two low OA) from the F_2 population derived from this cross. Crops of F_3 families obtained by self-pollination of these individuals were grown under a rainout shelter and subjected to a 30 day drought ending at anthesis. High OA families expressed greater OA at full turgor, estimated as the difference in osmotic potential between drought and control treatments, than the low OA families at the end of the drought period. Crops of high OA families extracted more water from the profile during the stress period, had greater shoot biomass and harvest index at physiological maturity, and greater grain yield (~30%). There was no effect of OA on these variables in the irrigated controls [122].

The significant value of OA as a key trait contributing to drought tolerance in sunflower prompted the analysis of QTL and the development of markers for this

trait. Jamaux *et al*. [123] identified both physiological and molecular markers of OA based on two pools of genotypes differing for the trait. Two families of sunflower lines with contrasting OA, T− (low desiccation rate) and T+ (high desiccation rate), differed significantly in relative water loss (RWL) of excised leaves under watered and dry conditions. Since the T−/T+ lines had contrasting OA, the RWL criterion could be considered a marker of OA, at least in the T−/T+ genetic background. The higher the values of OA, the lower were the RWL values. Differential screening of two cDNA libraries, one obtained from a T− and the other from a T+ nonstressed sunflower plant, led to the isolation of three constitutive clones, DRS12, DRS14, and DRS26. Although no DRS12- and DRS14-related protein was found in databases, the DRS26 cDNA showed a high sequence homology and identity with a mammalian amino acid transporter, suggesting that the DRS26 polypeptide could be involved in vacuolar transport of osmolytes such as amino acids. RFLP analysis with the restriction enzyme *Bam*HI and the DRS26 cDNA probe differentiated the two families of sunflower and suggested a role for DRS26/*Bam*HI as a marker of T− genotypes. When bulk analysis with RAPDs was conducted, a primer was found that easily differentiated T− and T+ individuals [123]. Using an integrated and high-density linkage map based on SSR and AFLPs, Poormohammad Kiani *et al*. [124] localized 8 QTLs for OA on a population of 129 RILs. Four of them were colocated with QTL for other plant water status variables. A major QTL for OA on LG 5 accounted for 29% of the phenotypic variation. These exciting results indicate not only that OA is a key trait for developing sunflower genotypes with increased tolerance to water deficits but also that this complex and technically difficult-to-assess trait can be approached by MAS. However, more research is needed for QTL confirmation and validation before its practical implementation in sunflower breeding programs.

47.4.5.2 **Leaf Expansion**

During the vegetative phase, and to maintain plant water status within tolerable limits, the sunflower crop relies more on restricting interception of radiation and hence evaporative demand than it does on stomatal closure, due to its explorative root system. The sensitivity of leaf expansion to water deficit has been demonstrated in a number of studies. The results showed that crops subjected to water deficits before anthesis develop small leaf area index (LAI), but maintain activity per unit leaf area [125–129]. Even in crops that are visibly wilted, stomata do not close completely and photosynthesis continues [130]. The effects of water stress on leaf expansion are mediated by changes in both cell number and cell size. The latter effect predominates in leaves unfolding early during stress episodes and the former in later stages of exposure to stress, consistent with the partial temporal separation of the processes of cell division and expansion [131].

The extent of leaf growth reduction caused by water deficit is very important in determining the adaptation of a certain crop variety to a climate scenario. In a scenario where long-term droughts are expected, a genotype that reduces its leaf growth is more likely to reach maturity with a certain amount of available water. On the other hand, in a scenario where short-term water deficits are expected, a genotype that maintains leaf growth is likely to have higher yields [132]. It has been shown that a

genotype can maintain its leaf area by maintaining growth rate [132] or by increasing the duration of leaf growth [133]. Moreover, an increased duration of growth could have the benefit of increasing the opportunity for recovery after rainfall [134]. The natural genetic variability for these traits could be used to develop crop varieties adapted to specific scenarios. Despite this, breeding for these traits is not a common approach for obtaining drought resistance in crop species, probably because of a lack of well-characterized sources of genetic variability. In this sense, the work reported by Pereyra-Irujo *et al.* [135] is an important first step to determine the feasibility of this approach in sunflower breeding. Eighteen nonbranching sunflower inbred lines, comprising most of the genetic variability of cultivated sunflower, were selected by these authors to analyze the response of leaf growth to water deficit in order to identify and quantitatively describe sources of genetic variability for this trait that could be used to develop sunflower varieties adapted to specific scenarios. Plants were subjected to long-term, constant-level, water deficit treatments [136] and the response to water deficit quantified by means of growth models at cell, leaf, and plant scale [137, 138]. Significant variation among lines was found for the response of leaf expansion rate and of leaf growth duration, with an equal contribution of these responses to the variability in the reduction of leaf area. Intrinsic genotypic responses of rate and duration at a cellular scale were linked to genotypic differences in whole-plant leaf area profile to water deficit. The results reported suggested that genetic differences in leaf growth rate under water deficit could be determined by cell wall properties, while increased duration of leaf growth is partly due to a prolonged phase of epidermal cell division. This implies that rate and duration responses could be the result of different physiological mechanisms and are therefore capable of being combined to increase the variability in leaf area response to water deficit in sunflower [135]. Identifying the mechanisms underlying genetic differences in the response of leaf growth to water deficit and exploring the genetic base of the crop for these mechanisms are of paramount importance as initial steps toward marker dissection of the relevant traits and their validation in different genetic backgrounds that, ultimately, will allow the implementation of this novel strategy in sunflower improvement for drought tolerance.

47.4.5.3 Cold Tolerance during Germination and Emergence

The anticipation of planting dates as a strategy to maximize the growing season and to escape drought stress during flowering or grain filling has increased the importance of low-temperature stress tolerance in sunflower during germination and early growth to increase yield potential and its stability [139, 140].

Even though it has been reported that growth of sunflower seedlings was inhibited to some degree when they were subjected to suboptimal temperatures [141], there exists genetic variability for cold tolerance during initial growth stages. Genotypic variability for emergence rate under field conditions during early planting and for germination rate at low temperatures under controlled conditions has been reported for a set of 13 commercial hybrids and 26 inbred lines [139]. Also, it has been shown that there exists variability in the cultivated gene pool for the relative crop growth rates under low-temperature conditions by

screening a group of 21 inbred lines under field conditions [140]. More recently, a study was conducted to identify physiological traits associated with cold tolerance in sunflower and to identify the genomic regions involved in their variation [142]. A population of 98 RILs and their two parents were sown in the field under the conventional sowing date (control) and 1 or 2 months earlier (long-term low-temperature treatments). Several traits putatively associated with cold tolerance and acclimation mechanisms to stress conditions have been investigated at early development stages. Significant differences were observed among the three sowing dates for all traits. Chlorophyll content and specific leaf area were genetically associated with cold tolerance, which suggests that they could be used as selection criteria in conventional breeding programs. QTL analyses show that several putative genomic regions are involved in the variation in the physiological traits studied under low-temperature conditions [142].

All these results indicated that there exist enough variability and phenotypic and molecular tools to breed sunflower for cold tolerance during initial stages of growth.

47.4.6 Traits that are Awaiting More Research

47.4.6.1 Stay Green

Delayed leaf senescence during the grain-filling phase of grain crops, or stay green (SG), may be functional, when the loss of canopy capacity for carbon fixation is delayed or occurs at a slower rate, or is cosmetic, when maintenance of leaf chlorophyll is combined with the disassembly of the photosynthetic apparatus [143, 144]. Functional SG has been recently demonstrated in sunflower and is considered a valuable trait in sunflower breeding since it may contribute positively to increases in yield potential through increments in biomass production or yield stability under conditions of water shortage, late sowings, or high plant population density [145]. Functional SG can also increase resistance to stalk breakage (stem lodging) by preventing (or minimizing) remobilization of carbohydrates from the stem during grain filling by maintaining crop photosynthesis [146]. Susceptibility to stem and root lodging in sunflower increases with crop population density suggesting that SG could be a valuable secondary trait in selection for higher and more stable yields in this crop species [147, 148].

Studies on variability and inheritance of the stay-green trait in sunflower using two crosses indicated that additive effects were the main source of genetic variation and the authors concluded that selection for this trait could be made in early-generation segregating populations [149]. However, the trait studied by them was stem color at maturity and not delayed leaf senescence.

Identification of sources for the functional SG trait in sunflower by exploring the genetic base of this crop will lead to a significant advance in breeding for yield potential and stability and greater adaptation to drought conditions. Also, it will allow the discovery of stay-green drought tolerance QTL or genes to speed up their introduction into elite genotypes, as was the approach in other crops [150–152].

47.4.6.2 Tolerance to Stem Lodging

Root and stem lodging, defined as the permanent displacement of the stem from its vertical position, causes important yield losses in sunflower. The prostrate head of lodged plants is not retrieved during mechanical harvesting causing significant losses. Also, lodging may contribute to fixing the upper limit to commercially viable crop population density since yield is known to increase up to densities higher than those used at present [148]. Sunflower lodging has been observed to result from failure of the root anchorage system or from tensile failure of the stems. The susceptibility to lodging and its occurrence in stems or roots of crops exposed to high winds depend on complex interactions between the mechanical properties of the stems and the soil–root system that anchors the plants, the shape of the upper sections of the plant that capture wind gusts, and rain. The values of these variables vary throughout crop development and can change with cultural practices, genotype, and soil properties. Stem lodging can occur in well-anchored crops when the force applied to the lower portion of the stem exceeds the stem failure moment. Root lodging is usually associated with rain that weakens the anchorage (i.e., the soil–root system) via a reduction in soil strength [147, 153]. Crops are particularly susceptible to stem lodging during grain filling and at harvest maturity, but the temporal and spatial unpredictability of lodging events under field conditions has hampered systematic research on this issue [148].

Sposaro *et al*. [154] have successfully adapted previous models for lodging in cereals to the sunflower crop, and this model has been shown to perform well under field conditions [148, 154]. It provides a systemic framework that can handle the multiple determinants of lodging and can serve to establish the relative importance of several plant variables critical to the determination of root and stem lodging susceptibility, providing useful guidelines for conventional breeding and for the dissection of this complex trait by molecular markers. The demonstration of the existence of genotypic differences in tolerance to root [147] and stem lodging [148] should encourage the exploration of the sunflower genetic base in order to identify sources of tolerance at high crop population densities in sunflower.

47.4.6.3 Reduced Height

Progress in improving the standability of conventional height sunflower has been slow [1]. Present hybrids, when protected from lodging and disease, show increase in yield potential with crop population densities almost three times the commercial density of 5 plants m^{-2} [155]; it seems very likely that propensity to lodge at high crop population densities also plays a part in reducing realizable yield potential in this crop [148]. Therefore, reduced height germplasm has the potential to increase both stem strength and yield potential of the sunflower crop.

Reduced height controlled by recessive genes in lines with a reduced number of leaves has been reported on several occasions [156–161]. However, none of them has been used to improve yield as yet because of the excessively severe phenotypes of these mutants. On the other hand, three sources of reduced height ("DDR", "Donsky," and "Donskoi 47") with an equal or similar number of leaves as conventional-height sunflowers were reported [162, 163]. DDR and Donsky were used to

develop several restorer and maintainer lines [164–166]. The inheritance of reduced plant height in the sunflower line Dw89, which traces back to "Donsky," was determined to be controlled by alleles at two loci, designated *dw1* and *dw2* [166]. Reduced height in Donskoi 47, on the other hand, is controlled by a single dominant gene, *Dw* [163]. The inheritance of dwarfism in the source DDR has not been determined. The utilization of traits for reduced plant height to maximize yield potential and reducing stem lodging in other crops [167, 168] is a strategy that deserves to be fully explored in sunflower.

47.5
Tolerance to Herbicides

Weeds compete with sunflower for moisture and nutrients, and depend on species for light and space. Weed competition causes substantial yield losses in sunflower, ranging from 20 to 70% [169–173]. The amount of yield reduction varies depending on the weed species, weed density, time of weed and crop emergence, climatic conditions, and type of soil. Competition can occur from early germinating weeds, such as species of winter annuals that germinate early in the spring and develop a competitive advantage if they emerge before the sunflower. Competition can be a serious problem under drought conditions, since several weeds have tolerance to limited moisture (e.g., *Kochia scoparia*), and even under cool temperature conditions following planting since sunflower emergence and initial growth are reduced, but these variables remain unaffected for many weed species [174].

Herbicides are the most desirable method for weed control; however, the availability of selective herbicides for the sunflower crop is quite limited and due to the high cost of herbicide registration, new molecules of herbicides are unlikely to be specifically developed for weed control in sunflower. For this reason, gene discovery and trait development for herbicide resistance in sunflower is one of the most important issues in raising the productivity and the competitive ability of this crop in the near future.

47.5.1
Nontarget-Site Herbicide Resistance

Herbicides can cause several injury problems to the sunflower crop (see, for example, Blamey *et al*. [174], Table 12–13, pp. 642–644). As a matter of fact, sunflower genotypes varied widely in their response to soil-applied and to postemergence herbicides [175]. For diclofop {2-[4-(2,4-dichlorophenoxy)phenoxy] propanoic acid), for example, genotypic response can range from susceptibility to tolerance and the tolerance level also varies according to growth stage of the plants, herbicide rate, and environmental conditions, such as temperature and relative humidity [176]. Natural variation in tolerance was recently investigated by screening 97 inbred lines with a combination of the herbicide imazamox and the insecticide Malathion, an inhibitor of cytochrome P450 monooxygenases (P450s). One tolerant line, named TolP450-1,

was selected and characterized in the field and in the greenhouse to evaluate its response to the herbicides imazamox, prosulfuron, and atrazine at different plant development stages (germination, VE, and V3) with and without Malathion. For all herbicides and all development stages analyzed, TolP450-1 showed significantly higher tolerance compared to the susceptible line RHA266, and in all cases the tolerance was reversed by Malathion [177]. The P450 gene family in plants encodes the most versatile class of enzymes involved in the metabolic detoxification of xenobiotics and in nontarget-site herbicide resistance in plants [178]. One of the first P450 genes identified for herbicide resistance, CYP76B1, was cloned from a sunflower relative, the Jerusalem artichoke, *H. tuberosus* [179, 180]. CYP76B1 metabolizes with high efficiency a wide range of xenobiotics, including alkoxycoumarins, alkoxyresorufins, and several herbicides of the class of phenylureas [181]. These observations indicate that there exists natural variation for P450s genes in the cultivated and wild sunflower germplasm to be used in developing new traits for nontarget herbicide tolerance for the sunflower crop.

47.5.2 Target-Site Herbicide Resistance

Imidazolinone and sulfonylurea herbicides have been demonstrated to have a broad spectrum of weed control activity, flexibility in timing of application, low usage rates, and low mammalian toxicity [182, 183]. These herbicides inhibit the enzymatic activity of acetohydroxyacid synthase (AHAS, EC 4.1.3.18, also known as acetolactate synthase, ALS [184, 185]), the first enzyme in the pathway for the synthesis of the branched chain amino acids valine, leucine, and isoleucine [186]. The same enzyme has been shown to be the site of action for the triazolopyrimidines [187], pyrimidyloxybenzoates [188], and sulfonylaminocarbonyl-triazolinones [189].

Imidazolinone- and/or sulfonilurea-tolerant plants with altered AHAS genes and enzymes have been discovered in many species, which permitted the development and commercialization of several herbicide-tolerant crops [183]. Resistance in most of these cases is due to a form of the AHAS large subunit enzyme (AHASL) that is less sensitive to herbicide inhibition and is conferred by a single, partially dominant nuclear gene [183, 190]. This reduction in herbicide binding is caused by mutations at key sites in the genes coding for the catalytic subunit of AHAS. Several authors have reviewed known mutations of the AHAS genes that confer tolerance to AHAS-inhibiting herbicides in plants [191, 192].

On the basis of molecular studies, Kolkman *et al.* [193] identified and characterized three genes coding for the AHAS catalytic subunits in sunflower (*Ahasl1*, *Ahasl2*, and *Ahasl3*). *Ahasl1* is a multiallelic locus and the only member of this small family where all the induced and natural mutations for herbicide resistance have been described thus far in sunflower. *Ahasl1-1* (also known as *Imr1* or Ar_{pur} [193, 194]) harbors a C-to-T mutation in codon 205 (*Arabidopsis thaliana* nomenclature) that confers a moderate resistance to imidazolinones, *Ahasl1-2* (also known as Ar_{kan}) shows a C-to-T mutation in codon 197 conferring high levels of sulfonylurea resistance [193], and *Ahasl1-3* presents a G-to-A mutation in codon 122 that confers high levels of imidazolinone resistance [195].

All these alleles are being used for the production of sunflower hybrids resistant to herbicides. The first commercial herbicide tolerance trait in sunflowers is known as "Imisun" and its development started in 1996, when imidazolinone-tolerant wild sunflowers were discovered in a field in Kansas, USA. Subsequent crossing of these plants with cultivated sunflower lines gave rise to imidazolinone-tolerant populations and lines [196] that were released as donor materials for developing hybrid varieties commercially launched in the United States, Argentina, and Turkey in 2004. The inheritance of Imisun is additively controlled by two genes, where one of them is the partially dominant allele *Ahasl1-1* and the other (Imr_2) is a modifier or enhancer factor [194, 197]. Synergistic effects of imidazolinones and Malathion on tolerance in Imisun genotypes suggest that *Imr2* is a member of the P450 gene family (Bulos and Sala, unpublished). To produce Imisun sunflower hybrids that express commercial tolerance levels to imidazolinone herbicides, both components need to be homozygous in the final variety. The second imidazolinone tolerance trait in sunflowers, known as CLPlus, is controlled by the expression of the partially dominant nuclear allele *Ahasl1-3* that was developed by seed mutagenesis and selection with imazapyr [198]. Biochemical studies together with the results of several years of evaluation under field conditions in many countries permit to conclude that CLPlus provides a better level of tolerance to imidazolinones than Imisun [199]. Owing to the high levels of tolerance, only one homozygous component, namely, *Ahasl1-3*, or the combination of both *Ahasl1-1* and *Ahasl1-3* alleles in the final hybrid variety, is required to achieve commercial tolerance levels [199, 200].

Sulfonylurea-tolerant sunflowers were developed from wild sunflower populations discovered in the United States [201]. The tolerance allele *Ahasl1-2* was introgressed into cultivated sunflower by forward crossing and selection with the herbicide tribenuron, and gave rise to the trait known as "Sures" [202]. The same type of tolerance was obtained by EMS mutagenesis [203] and was developed and commercialized under the name "ExpressSun" [204].

Introgression of genes for herbicide resistance into high-yield sunflower germplasm is being facilitated by MAS with diagnostic markers for each one of the resistance alleles [193, 205]. Selection of cultivated germplasm, wild *Helianthus* species, and mutagenized libraries will allow the discovery of new sources of herbicide resistance (e.g., [206]), especially other modes of action apart from the inhibition of AHAS in order to complement the current technologies.

47.6
Candidate Gene Approach

A large number of abiotic stress-induced genes have been identified in a wide range of plant species although a molecular basis for plant tolerance to these stresses remains far from being completely understood. Some examples of candidate genes obtained as a result of transcriptomic analysis and that plausibly play a relevant role in stress tolerance in sunflower have been reported, and they are described below.

47.6.1 Dehydrins

Late embryogenesis-abundant proteins are extremely hydrophilic proteins that were first identified in land plants. Intracellular accumulation is tightly correlated with acquisition of desiccation tolerance, and data support their capacity to stabilize other proteins and membranes during drying, especially in the presence of sugars such as trehalose. Among the water stress-induced proteins so far identified, dehydrins, the D-11 subgroup of late-embryogenesis abundant proteins [207], are frequently observed. Dehydrins are highly abundant in desiccation-tolerant seed embryos and accumulate during periods of water deficit in vegetative tissues. These proteins display particular structural features such as the highly conserved Lys-rich domain predicted to be involved in hydrophobic interaction leading to macromolecule stabilization [208, 209]. These drought-induced proteins lack a fixed three-dimensional structure. Their specific molecular action, as well as the reason for their disordered character, is as yet poorly understood. It has been speculated, however, that dehydrins are tuned to acquire a biologically active structure only under the conditions in which they normally function (i.e., upon dehydration) [210]. Very little is known about dehydrin functions *in planta*. Studies have established correlations between drought adaptation and dehydrin accumulation in several species. Positive correlations were also reported for species tolerant to stresses that have a dehydrative component such as salt stress [211, 212] and freezing and cold stress [209, 213–215].

To investigate correlations between phenotypic adaptation to water limitation and drought-induced gene expression, Cellier *et al.* [216] studied a model system consisting of a drought-tolerant line (R1) and a drought-sensitive line (S1) of sunflower subjected to progressive drought. R1 tolerance is characterized by the maintenance of shoot cellular turgor. Drought-induced genes (*HaElip1*, *HaDhn1*, and *HaDhn2*) were previously identified in the tolerant line. *HaDhn1*- and *HaDhn2*-deduced proteins belong to the dehydrin family, and *HaElip1* is a related homologue of early light-induced protein (ELIP) [217]. The accumulation of the corresponding transcripts was compared as a function of soil and leaf water status in R1 and S1 plants during progressive drought. In leaves of R1 plants, the accumulation of *HaDhn1* and *HaDhn2* transcripts, but not *HaElip1* transcripts, was correlated with the drought-adaptive response. Drought-induced abscisic acid (ABA) concentration was not associated with the varietal difference in drought tolerance. Stomata of both lines displayed similar sensitivity to ABA. ABA-induced accumulation of *HaDhn2* transcripts was higher in the tolerant than in the sensitive genotype. *HaDhn1* transcripts were similarly accumulated in the tolerant and in the sensitive plants in response to ABA, suggesting that additional factors involved in drought regulation of *HaDhn1* expression might exist in tolerant plants. In leaves of R1 plants, the steady-state level of each transcript increased gradually as the water potential declined. In the S1 plants, the fluctuations of the steady-state level of *HaElip1* transcripts were not correlated with the decreases in leaf water potential. Similar levels of *HaElip1* transcripts were accumulated in S1 and R1 plants except in leaves with water potentials between 20.9 and 21.2 MPa. Steady-state levels of *HaDhn1* and

HaDhn2 transcripts in S1 plants remained low and constant in leaves with a water potential of less than 20.6 MPa. At an equivalent water potential, they accumulated at a higher level in R1 than in S1 leaves. At low leaf water potential, steady-state levels of *HaDhn1* and *HaDhn2* transcripts were nine- and fivefold higher, respectively, in leaves of R1 compared to S1 plants [216]. Sequence analysis of the deduced dehydrin (Dhn1) proteins showed diversity in cultivated and wild sunflower accessions, the latter being more variable [218, 219]. Despite these exciting findings, no other study concerning mapping or validation of these genes for their role in drought tolerance was reported.

47.6.2 Tanscription Factors

As was described in Section 47.4.5, a large array of genes are activated by stress conditions, meaning several proteins are produced to join the pathways that subsequently lead to synergistic enhancement of stress tolerance [220–228]. These genes are classified into two groups: regulatory genes and functional genes. The regulatory group includes genes encoding various transcription factors (TFs), which can regulate various stress-inducible genes cooperatively or separately and may constitute gene networks. The functional group contains genes encoding metabolic components such as sugar, sugar alcohols, and amines, which play an important role in stress tolerance. Gaining an understanding of the mechanisms that regulate the expression of these genes is a fundamental issue in plant biology and will be necessary for the genetic improvement of plants for abiotic stress tolerance.

Stress tolerance seems to be controlled mostly at the transcriptional level [229], where the main players are proteins called transcription factors or *trans*-acting elements. Transcription factors are able to enhance or reduce the rate of transcription of their target genes. They specifically recognize and interact with DNA sequences (*cis*-acting elements) located in the regulatory regions of their targets. These TFs and *cis*-motifs function not only as molecular switches for gene expression but also as terminal points of signal transduction in the signaling processes. Typically, the TFs contain a distinct type of DNA binding domain and transcriptional regulation region, and are capable of activating or repressing transcription of multiple target genes [230, 231]. In plants, approximately 7% of the genome encodes for putative TFs [232]. It has been estimated that *Arabidopsis* and rice have between 1300 and 1500 transcription factor encoding genes [233, 234]. Some of them have been identified as stress responsive. Each of these stress-related transcription factor families exhibit a distinctive DNA binding domain [235].

Hahb-4 is a member of sunflower subfamily I of homeodomain-leucine zipper proteins, which constitutes a family of transcription factors found only in plants. *Hahb-4* was found to be regulated at the transcriptional level by water availability and abscisic acid [236]. Transgenic *A. thaliana* plants that constitutively overexpress *Hahb-4* developed shorter stems and internodes, rounder leaves, and more compact inflorescences than their nontransformed counterparts. Transgenic plants were more tolerant to water stress conditions, showing improved

development, a healthier appearance, and higher survival rates than wild-type plants [237]. Indeed, either under normal or drought conditions, they produce approximately the same seed weight per plant as wild-type plants under normal growth conditions. It has been proposed that Hahb-4 may participate in the regulation of the expression of genes involved in developmental responses of plants to desiccation and that it is a component of ethylene signaling pathways inducing a marked delay in senescence [238]. Furthermore, transgenic plants expressing this gene under the control of its own inducible promoter showed that the expression of this TF is regulated by external factors such as drought, extreme temperatures, osmotic stresses, and illumination conditions, and is specific to different tissues and organs of the plant. Their role as transcription factors is related to developmental events in response to such environmental conditions, particularly those in which abiotic factors generate stress [239–244]. Interestingly, this family of TFs was found only in sunflower and for this reason, it can be a useful candidate for obtaining transgenics with enhanced resistance to abiotic stress in other crops (see Section 47.8).

Another TF from sunflower is *HAhb-10*, a member of subfamily II homeodomain-leucine zipper proteins, whose expression is regulated by illumination conditions in photosynthetic tissues, and their function in plant development is associated with this environmental factor, particularly in the case of the shade avoidance response [245–247]. Transformed plants of *Arabidopsis* overexpressing HAhb-10 have a shorter time to maturity maintaining their seed yield [248], and also showed increased tolerance to oxidative stress produced by paraquat [248, 249].

47.6.3
Other Genes

A cDNA microarray containing about 800 clones covering major metabolic and signal transduction pathways allowed to identify many differentially expressed genes in leaves and embryos of drought-tolerant and -sensitive genotypes of sunflower subjected to water-deficit under field conditions [250]. The majority of the cDNA clones differentially expressed under water stress was found to display opposite gene expression profiles in a drought-tolerant genotype when compared to a drought-sensitive one. These dissimilarities suggest that the difference between tolerant and nontolerant plants is mainly associated with changes in mRNA expression. However, phenotypic variation resides also in changes in allelic sequences that can affect the efficiency of the encoded proteins. Hence, sequence variability of stress-related genes can modulate the stress response within a species.

Differential expression of four water stress-associated genes, aquaporin, dehydrin, leafy cotyledon1-like protein, and fructose-1,6 bisphosphatase, was examined using four RILs and parental lines presenting contrasting responses to dehydration and rehydration [114]. Water stress revealed a high genetic variability for water status and gas exchange parameters compared to well-watered genotypes. QTL mapping showed that RILs carrying different genomic regions for some QTL also presented physiologically different characteristics and gene expression patterns. The expres-

sion level of aquaporin genes in leaves of four RILs and their parents was downregulated by water stress and was associated with relative water content. Downregulation was also associated with genomic regions having alleles with negative effects on plant water status. The level of dehydrin transcripts, on the other hand, increased in leaves of all studied RILs in response to water stress. Transcript accumulations of dehydrin and leafy cotyledon1-like genes, likely involved in protective tolerance processes, were not correlated directly with plant water status or QTL effects. Downregulation of fructose-1,6 bisphosphatase was observed under water stress. Net photosynthesis rate and the fructose-1,6 bisphosphatase gene expression levels were associated mainly after rehydration [114]. These results indicate that there exists an association between physiological response to water stress and differential expression of water stress-related genes.

Sequence polymorphisms of eight unique genes putatively involved in drought response in eight inbred lines of sunflower of different origin and phenotypic characters and showing different drought response in terms of leaf RWC were analyzed. The selected genes encode a dehydrin, a heat shock protein, a nonspecific lipid transfer protein, a z-carotene desaturase, a drought-responsive element binding protein, a NAC-domain transcription regulator, an auxin binding protein, and an ABA-responsive C5 protein. A pairwise comparison between genetic distances calculated on the eight genes and the difference in RWC showed a significant correlation in the first phases of drought stress [219].

These initial results concerning sequence variability for putative stress-related sequences encourage more research in this area, using a broader and diverse set of lines, coupled with association mapping and extensive phenotyping. The likelihood of this approach for MAS will depend on the confirmation and validation of the selected candidate genes in modifying the stress response on different genetic backgrounds.

47.7 Marker-Assisted Selection

The fundamental advantages of MAS over conventional phenotypic selection that can be exploited by breeders to accelerate the breeding process are as follows: (a) MAS may be simpler than phenotypic screening, which can save time, resources, and effort; (b) selection can be carried out at the seedling stage; and (c) single plants can be selected [251, 252].

In previous sections, we described numerous studies on DNA markers linked to genes or QTL for different traits related to yield potential or tolerance to abiotic stresses. In contrast, literature on practical application of these markers in sunflower breeding programs remains very limited. Even though it was assumed that most markers associated with QTL from preliminary mapping studies were directly useful in MAS, it has become widely accepted that QTL confirmation, QTL validation, and/or fine (or high resolution) QTL mapping may be required. Although there are examples of highly accurate preliminary QTL mapping data as determined by

subsequent QTL mapping research [253], ideally a confirmation step is required because QTL positions and effects can be inaccurate due to factors such as sampling bias [254]. QTL validation refers to the verification that a QTL is effective in different genetic backgrounds [255]. Additional marker-testing steps may involve identifying markers within a 10 cM "window" spanning and flanking a QTL and converting markers into a form that requires simpler methods of detection [251, 256].

Ideal markers for MAS are those based on gene mutations underlying the trait of interest. This kind of markers has been developed in sunflower for oil quality traits (e.g., [108]) and resistance to herbicides [193, 205]. For traits related to yield, yield components, or tolerance to abiotic stresses, the situation is more complex. Factors such as population structure and size, parental selection and genetic background effects, epistasis, inaccurate phenotyping, or QTL × environment interactions contribute to bias the estimation of QTL effects, thus reducing the likelihood of successful use of these QTL in MAS programs [15, 257, 258].

QTL validation in independent samples and in different genetic backgrounds and environments is, therefore, necessary before using them in MAS breeding programs. A major challenge that remains is to confirm that QTL discovered in a given mapping population will improve yield potential or drought tolerance when introduced into high-yield elite genotypes. This is particularly difficult when the traits are governed by "context-dependent" gene effects (i.e., interaction with other genes and/or environment). In these cases, the value of the QTL alleles can differ depending on the genetic structure of the current germplasm set in the breeding program [259]. Also, a desirable QTL allele discovered in nonelite genetic material may not offer any improvement because the allele may already be ubiquitous in present varieties. In addition, the effects of the positive allele may not be transferable to elite backgrounds due to unfavorable epistatic interactions [260].

Taking into account all these considerations, there exist examples of successful implementation of MAS for yield-related traits in sunflower. QTL for oil content, for example, have been validated across generations, environments, and mapping populations [77, 108] and some of the QTL underlying differences between genotypes have been associated with phenotypic markers (*Hyp*, hypodermal pigment [77, 80, 108]), which allowed León *et al*. [261] to establish combined marker and phenotypic-assisted selection for high oil content during the backcross process.

47.8
Transgenic Breeding

Tissue culture and plant regeneration are key steps of the transformation process. Therefore, sunflower biotechnologists invest considerable effort in this area. Based on the tissue type for regeneration, sunflower culture systems include somatic and zygotic embryos, hypocotyls, mature cotyledons, and protoplasts (see Ref. [262] for review). The regeneration rate of whole sunflower plants is variable and depends on genotypes used, explant type, and development stage, and culture media and conditions [263]. Genetic variation is closely associated with regeneration ability.

Organogenetic traits were mapped to QTL by Deglene *et al.* [264] and Flores Berrios *et al.* [265]. Some segments of the LG 1, 15, and 17 are likely to contain genes important for organogenesis, somatic embryogenesis, and protoplast division. The QTL identified in these three LGs should be involved in cell division in early events associated with cell differentiation [266].

Sunflower genetic transformation was achieved by *Agrobacterium tumefaciens*-mediated protocols [267–269] and by combining this technique with particle bombardment [270], sonication [271], wounding with glass beads [272], or enzymatic treatments [271, 273]. Most of the published protocols of sunflower transformation showed a low efficiency [268, 270, 273–279]. Efficient transformation protocols with high reproducibility and high transformation frequency have been reported [280, 281] together with techniques to enhance the selection of transformed explants [279]. The usefulness of these methodologies remains to be assessed. On the other hand, an optimized protocol for *Agrobacterium*-mediated transient transformation of sunflower leaf disks was developed with the aim of quickly overexpressing or silencing a given gene, enabling the study of several biochemical processes and the characterization of sunflower regulatory sequences [282].

Most of the transgenic traits in sunflower under field trials at present are focused on biotic stress and herbicide resistances. Cantamutto and Poverene [283] mentioned several examples of them, including tolerance to glyphosate and glufosinate ammonium and resistance to insects, fungal diseases, and broomrape, and quality traits such as enhanced protein quality and modified stereate content. They were developed mainly by private companies, but some of them were obtained also from public institutes [278, 284, 285]. Traits related to abiotic stresses in sunflower using the transgenic approach remain to be developed.

Although transgenic sunflower varieties have already been obtained, and they remain the subject of ongoing research in both the United States and Argentina, the interest in GM sunflower research has decreased in the twenty-first century, mainly because official control bureaus have imposed restrictions in the face of ecological concerns [283]. These concerns are related to the risks associated with the gene flow from transgenic cultivars to the wild flora and are a matter of general controversy not only in the United States but also in other parts of the world [286]. Gene flow from domesticated, transgenic, or mutant sunflower plants to wild species is well documented [287–290]. This potential gene containment is discouraging the advancement and realization of transgenics' full development in sunflower.

In spite of the low transformation efficiency and biosafety concerns related to transgenic sunflower, the use of sunflower genes to mitigate abiotic stresses in other crops has been described. A xenobiotic-inducible cytochrome P450 gene, CYP76B1, obtained from *H. tuberosus* was constitutively expressed in tobacco and *Arabidopsis* conferring to the transformed plants an enhanced resistance to several herbicides and xenobiotics. Beside increased herbicide tolerance, expression of CYP76B1 has no other visible phenotype in the transgenic plants and can be a potential tool for phytoremediation of contaminated soils [291]. Sunflower transcription factors, like those described in Section 47.6, have been proposed as candidates for genetic

transformation of different crops in order to enhance the tolerance not only to abiotic stresses but also to modify the growth cycle and the tolerance to xenobiotics in transgenic plants [248, 249].

47.9 Prospects

Improvements in sunflower yield potential and stability during the last decades have been slow and mainly based on the unconscious pyramiding of yield associated characters with biotic and abiotic stress-related traits. Research has led to three main approaches to change the objectives and the current tools for sunflower breeding. First of all, plant physiology provided new tools and models to understand the complex network of yield and stress-related traits in order to identify target traits useful for improving selection efficiency. Second, molecular genetics has led to the discovery of a large number of loci affecting yield under stress conditions or the expression of stress tolerance-related traits. Third, molecular biology has provided genes that are useful as candidate sequences to dissect QTL or are useful for transgenic approaches.

Even though there exist some successful results of synergistic interactions among these three approaches, the great challenge in the near future is to formalize the integration of molecular genetics with physiology and breeding in order to (a) identify the most relevant traits as targets for research, (b) screen the genetic base of the sunflower crop to detect the most promising genotypes as putative sources for these traits, (c) establish and screen introgression libraries from wild species of *Helianthus* that are reservoirs of potential useful genes for stress tolerance [292], and (d) dissect yield and other integrative traits that influence stress tolerance into heritable traits by using phenotyping platforms with model-assisted methods [293–296]. Routine cloning of the genes underlying the QTL is still a long way off, but it will, ultimately, provide simple markers for an effective MAS.

To date most plant QTL have been cloned by the positional cloning approach, although alternative strategies based on candidate genes and linkage disequilibrium may represent an interesting shortcut to QTL cloning [258, 297]. Cloning genes for stress-related traits offers the opportunity to approach stress tolerance by means of reverse genetics. So far, the utilization of mutagenesis as a source of new traits for increasing stress tolerance has been unaffordable. However, reverse genetics coupled with a tilling strategy and an efficient phenotyping platform could be a virtual inexhaustible source of new useful allelic variants for increasing stress tolerance.

Ultimately, as was pointed out by Collins *et al.* [258], integration of QTL information into a breeding pipeline aimed at improving tolerance to abiotic stresses will best be achieved within a multidisciplinary context able to provide the operational framework required to correctly link the stress-responsive mechanisms of crops with the functional variation of the relevant networks at the cellular and molecular levels.

References

1 Miller, J.F. and Fick, G.N. (1997) The genetics of sunflower, in *Sunflower Technology and Production, Agronomy Series* (ed. A.A. Schneiter), ASA, CSSA and SSSA, Madison, WI, USA, pp. 441–496.

2 Sánchez Muñiz, F.J. and Cuesta, C. (2003) Sunflower oil, in *Encyclopedia of Food Sciences and Nutrition*, 2nd edn, pp. 5672–5680.

3 Bunta, G. and Mario, B. (2008) The first results regarding the breeding of some sunflower hybrids for biodiesel. Analele Univ. din Oradea, Fascicula: Protectia Mediului, XIII 33–38.

4 Gulya, T.J. (2004) Sunflower, in *Encyclopedia of Grain Science*, Academic Press, pp. 264–270.

5 Rieseberg, L.H. and Seiler, G.J. (2001) Molecular evidence and the origin and development of the domesticated sunflower (*Helianthus annuus*, Asteraceae). *Econ. Bot.*, **44**, 79–91. doi: 10.1007/BF02860477

6 Harter, A.V., Gardner, K.A., Falush, D., Lentz, D.L., Bye, R.A., and Rieseberg, L.H. (2004) Origin of extant domesticated sunflowers in eastern North America. *Nature*, **430**, 201–205.

7 Wills, D.M. and Burke, J.M. (2006) Chloroplast DNA variation confirms a single origin of domesticated sunflower (*Helianthus annuus* L.). *J. Hered.*, **97** (4), 403–408.

8 Heiser, C.B., Smith, D.M., Clevenger, S.B., and Martin, W.C. (1969) The North American sunflowers (*Helianthus*). *Mem. Torrey Bot. Club*, **22**, 1–218.

9 Tang, S. and Knapp, S.J. (2003) Microsatellites uncover extraordinary diversity in native American landraces and wild populations of cultivated sunflower. *Theor. Appl. Genet.*, **106**, 990–1003.

10 Liu, A. and Burke, J.M. (2006) Patterns of nucleotide diversity in wild and cultivated sunflower. *Genetics*, **173**, 321–330.

11 Gundaev, A.I. (1971) Basic principles of sunflower selection, in *Genetic Principles of Plant Selection*, Nauka, Moscow, pp. 417–465.

12 Leclercq, P. (1969) Une sterilité cytoplasmique chez le tournesol. *Ann. Amelior. Plant.*, **19**, 99–106.

13 Kinman, M.L. (1970) New developments in the USDA and state experiment station sunflower breeding programs. Proceedings of the 4th International Sunflower Conference, Memphis, TN, USA, pp. 181–183.

14 Skoric, D. (1992) Achievements and further directions of sunflower breeding. *Field Crops Res.*, **30**, 231–270.

15 Fernández-Martínez, J.M., Pérez-Vich, B., and Velasco, L. (2009) Chapter 6: Sunflower, in *Oil Crops, Handbook of Plant Breeding 4* (eds J. Vollmann and I. Rajcan), Springer Science + Business Media, pp. 155–232.

16 Cheres, M.T. and Knapp, S.J. (1998) Ancestral origins and genetic diversity of cultivated sunflower: coancestry analysis of public germplasm. *Crop Sci.*, **38**, 1476–1482.

17 Kolkman, J.M., Berry, S.T., Leon, A.J., Slabaugh, M.B., Tang, S., Gao, W., Shintani, D.K., Burke, J.M., and Knapp, S.J. (2007) Single nucleotide polymorphisms and linkage disequilibrium in sunflower. *Genetics*, **177**, 457–468.

18 Panchenco, A.Y. (1966) Sunflower production and breeding in the USSR. Proceedings of the 2nd International Sunflower Conference, Morden, Manitoba, Canada, pp. 15–29.

19 Fick, G.N. and Miller, J.F. (1997) Sunflower breeding, in *Sunflower Production and Technology, Agronomy Series* (ed. A.A. Schneiter), ASA, CSSA and SSSA, Madison, WI, USA, pp. 395–439.

20 Vrânceanu, A.V. and Stoenescu, F. (1971) Pollen fertility restorer gene from cultivated sunflower (*Helianthus annuus* L.). *Euphytica*, **20**, 536–541.

21 López Pereira, M., Sadras, V.O., and Trápani, N. (1999) Genetic improvement of sunflower in Argentina between 1930 and 1995. I. Yield and its components. *Field Crops Res.*, **62**, 157–166.

22 López Pereira, M., Sadras, V.O., and Trápani, N. (1999) Genetic improvement of sunflower in Argentina between 1930 and 1995. II. Phenological development, growth and source–sink relationship. *Field Crops Res.*, **63**, 247–254.

23 López Pereira, M., Sadras, V.O., and Trápani, N. (2000) Genetic improvement of sunflower in Argentina between 1930 and 1995 III. Dry matter partitioning and grain composition. *Field Crops Res.*, **67**, 215–221.

24 Sadras, V.O., Trápani, N., Pereyra, V.R., López Pereira, M., Quiroz, F., and Mortarini, M. (2000) Intraspecific competition and fungal diseases as sources of variation in sunflower yield. *Field Crops Res.*, **67**, 51–58.

25 de la Vega, A.J. and Chapman, S.C. (2010) Mega-environment differences affecting genetic progress for yield and relative value of component traits. *Crop Sci.*, **2** (50), 574–583.

26 Slafer, G.A., Satorre, E.H., and Andrade, F.H. (1993) Increases in grain yield in wheat from breeding and associated physiological changes, in *Genetic Improvement of Field Crops* (ed. G.A. Slafer), Marcel Dekker, New York, pp. 1–68.

27 Duvick, D.N. and Cassman, K.G. (1999) Post-green revolution trends in yield potential of temperate maize in the north-central United States. *Crop Sci.*, **39**, 1622–1630.

28 Bell, M.A., Fischer, R.A., Byerlee, D., and Sayre, K. (1995) Genetic and agronomic contributions to yield gains: a case study for wheat. *Field Crops Res.*, **44**, 55–65.

29 Bertero de Romano, A., Vázquez, A., Piubello, S., and Sala, C.A. (1994) Quantification of the relationship between verticillium wilt intensity and loss in yield of sunflower (*Helianthus annuus* L.), and feasibility of utilizing the hypodermic inoculation technique as a selection method. *Helia*, **17** (20), 49–54.

30 Bertero de Romano, A., Vázquez, A., Piubello, S., and Sala, C.A. (1994) La verticilosis del girasol en Argentina. *Revista Oleaginosos* (2), 124–127.

31 Frensham, A.B., Barr, A.R., Cullis, B.R., and Pelham, S.D. (1998) A mixed model analysis of 10 years of oat evaluation data: use of agronomic information to explain genotype by environment interaction. *Euphytica*, **99**, 43–56.

32 de la Vega, A.J., DeLacy, I.H., and Chapman, S.C. (2007) Progress over 20 years of sunflower breeding in central Argentina. *Field Crops Res.*, **100**, 61–72.

33 Allard, R.W. and Bradshaw, A.D. (1964) Implication of genotype-environment in applied plant breeding. *Crop Sci.*, **4**, 503–507.

34 Lin, C.S. and Binns, M.R. (1988) A method of analyzing cultivar×location×year experiments: a new stability parameter. *Theor. Appl. Genet.*, **73** (3), 425–430.

35 de la Vega, A.J., Chapman, S.C., and Hall, A.J. (2001) Genotype by environment interaction and indirect selection for yield in sunflower. I. Two mode pattern analysis of oil and biomass yield across environments in Argentina. *Field Crop Res.*, **72**, 17–38.

36 Chapman, S.C. and de la Vega, A.J. (2002) Spatial and seasonal effects confounding interpretation of sunflower yields in Argentina. *Field Crop Res.*, **73**, 107–120.

37 Maas, E.V. and Hoffman, G.J. (1977) Crop salt tolerance, current assessment. *J. Irrig. Drain. Div.*, **103**, 115–134.

38 Francois, L.E. (1996) Salinity effects on four sunflower Hybrids. *Agron. J.*, **88**, 215–219.

39 Hussain, M.K. and Rehman, O.U. (1997) Evaluation of sunflower *(Helianthus annuus* L.) germplasm for salt tolerance at the shoot stage. *Helia*, **20**, 69–78.

40 Ashraf, M. and Tufail, M. (1995) Variation in salinity tolerance in sunflower (*Helianthus annuus* L.). *J. Agron. Crop Sci.*, **174** (5), 351–362.

41 Ashraf, M., Zafar, Z.U., and O'Learly, J.W. (1995) Genetic variation for salt tolerance in sunflower (*Helianthus annuus* L.). *Hereditas*, **123**, 141–145.

42 Welch, M.E. and Rieseberg, L.H. (2002) Habitat divergence between a homoploid hybrid sunflower species, *Helianthus paradoxus* (Asteraceae), and its progenitors. *Am. J. Bot.*, **89**, 472–478.

43 Lexer, C., Welch, M.E., Durphy, L., and Rieseberg, L.H. (2003) Natural selection

for salt tolerance quantitative trait loci (QTLs) in wild sunflower hybrids: implications for the origin of *Helianthus paradoxus*, a diploid hybrid species. *Mol. Ecol.*, **12**, 1225–1235.

44 Lexer, C., Lai, Z., and Rieseberg, L.H. (2003) Candidate gene polymorphisms associated with salt tolerance in wild sunflower hybrids: implications for the origin of *Helianthus paradoxus,* a diploid hybrid species. *New Phytol.*, **161**, 225–233.

45 Karrenberg, S., Edelist, C., Lexer, C., and Rieseberg, L.H. (2006) Response to salinity in the homoploid hybrid species *Helianthus paradoxus* and its progenitors *H. annuus* and *H. petiolaris. New Phytol.*, **170**, 615–629.

46 Edelist, C., Raffoux, X., Falque, M., Dillmann, C., Sicard, D., Rieseberg, L.H., and Karrenberg, S. (2009) Differential expression of candidate salt-tolerance genes in halophyte *Helianthus paradoxus* and its glycophyte progenitors *H. annuus* and *H. petiolaris* (Asteraceae). *Am. J. Bot.*, **96** (10), 1830–1838.

47 Seiler, G.J. (1991) Registration of 13 downy mildew tolerant interspecific sunflower germplasm lines derived from wild annual species. *Crop Sci.*, **31**, 1714–1716.

48 Miller, J.F. and Seiler, G.J. (2003) Registration of five oilseed maintainer (HA 429–HA 433) sunflower germplasm lines. *Crop Sci.*, **43**, 2313–2314.

49 Fereres, E., Giménez, C., and Fernández, J.M. (1986) Genetic variability in sunflower cultivars under drought I. Yield relationships. *Aust. J. Agric. Res.*, **37**, 573–582.

50 Giménez, C. and Fereres, E. (1986) Genetic variability in sunflwer cultivars under drought II. Growth and water relations. *Aust. J. Agric. Res.*, **37**, 583–597.

51 Connor, D.J. and Hall, A.J. (1997) Sunflower physiology, in *Sunflower Technology and Production, Agronomy Series* (ed. A.A. Schneiter), ASA, CSSA, and ASSA, Madison, WI, USA, **35**, pp. 1–19.

52 Škorić, D. (2009) Sunflower breeding for resistance to abiotic stresses. *Helia*, **32** (50), 1–15.

53 Ludlow, M.M. and Muchow, R.C. (1990) A critical evaluation of traits for improving crop yield in water-limited environments. *Adv. Agron.*, **43**, 107–153.

54 Barker, M.S., Kane, N.C., Matvienko, M., Kozik, A., Michelmore, R.W., Knapp, S.J., and Rieseberg, L.H. (2008) Multiple paleopolyploidizations during the evolution of the Compositae reveal parallel patterns of duplicate gene retention after millions of years. *Mol. Biol. Evol.*, **25** (11), 2445–2455.

55 Paniego, N., Heinz, R., Fernández, P., Talia, P., Nishinakamasu, V., and Hopp, H.E. (2007) Sunflower, in *Genome Mapping and Molecular Breeding in Plants, Vol. 2, Oilseeds* (ed. C. Kole), Springer, Berlin, pp. 153–177.

56 Berry, S.T., Leon, A.J., Hanfrey, C.C., Challis, P., Burkholz, A., Barness, S., Rufener, G.K., Lee, M., and Caligari, P.D.S. (1995) Molecular marker analysis of *Helianthus annuus* L.: 2. Construction of an RFLP linkage map for cultivated sunflower. *Theor. Appl. Genet.*, **91**, 195–199.

57 Gentzbittel, L., Vear, F., Zhang, Y.X., and Berville, A. (1995) Development of a consensus linkage RFLP map of cultivated sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.*, **90**, 1079–1086.

58 Berry, S.T., Leon, A.J., Peerbolte, R., Challis, C., Livini, C., Jones, R., and Feingold, S. (1997) Presentation of the Advanta sunflower RFLP linkage map for public research. Proceedings of the 19th Sunflower Research Workshop, 9–10 Jan. 1997, Fargo, ND pp. 113–118.

59 Jan, C.C., Vick, B.A., Miller, J.F., Kahler, A.L., and Butler, E.T., III. (1998) Construction of an RFLP linkage map for cultivated sunflower. *Theor. Appl. Genet.*, **96**, 15–22.

60 Rieseberg, L.H. (1998) Genetic mapping as a tool for studying speciation, in *Molecular Systematics of Plants*, 2nd edn (eds D.E. Soltis, P.S. Soltis, and J.J. Doyle), Chapman and Hall, New York, pp. 459–487.

61 Gentzbittel, L., Mestries, E., Mouzeyar, S., Mazeyrat, F., Badaoui, S., Vear, F., Tourvieille de Labrouhe, D., and Nicolas, P. (1999) A composite map of expressed

sequences and phenotypic traits of the sunflower *Helianthus annuus* L. genome. *Theor. Appl. Genet.*, **99**, 218–234.

62 Berry, S.T., Leon, A.J., Challis, P., Livin, C., Jones, R., Hanfrey, C.C., Griffiths, S., and Roberts, A. (2003) Construction of a high density, composite RFLP linkage map for cultivated sunflower *Helianthus annuus*. Proceedings of International Sunflower Conference, 12–20 June, 1996, Beijing, China, pp. 1150–1160.

63 Knapp, S.J., Berry, S., and Rieseberg, L.H. (2001) Genetic mapping in sunflowers, in *DNA Markers in Plants* (eds R.L. Phillips and I.K. Vasil), Kluwer, Dordrecht, The Netherlands, pp. 379–403.

64 Lawson, W.R., Goulter, K.C., Henry, R.J., Kong, G.A., and Kochman, J.K. (1998) Marker-assisted selection for two rust resistance genes in sunflower. *Mol. Breed.*, **4**, 227–234.

65 Lu, Y.H., Melero-Vara, J.M., García-Tejada, J.A., and Blanchard, P. (2000) Development of SCAR markers linked to the gene Or5 conferring resistance to broomrape (*Orobanche cumana* Wallr.) in sunflower. *Theor. Appl. Genet.*, **100**, 625–632.

66 Peerbolte, R.P. and Peleman, J. (1996) The Cartisol sunflower RFLP map (146 loci) extended with 291 AFLP markers. Proceedings of the 18th Sunflower Research Workshop, 11–12 Jan. 1996, Fargo, ND, pp. 174–178.

67 Gedil, M.A., Wye, C., Berry, S., Segers, B., Peleman, J., Jones, R., Leon, A., Slabaugh, M.B., and Knapp, S.J. (2001) An integrated restriction fragment length polymorphism-amplified fragment length polymorphism linkage map for cultivated sunflower. *Genome*, **44**, 213–221.

68 Al-Chaarani, G.R., Gentzbittel, L., Huang, X.Q., and Sarrafi, A. (2004) Genotypic variation and identification of QTLs for agronomic traits, using AFLP and SSR markers in RILs of sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.*, **109**, 1353–1360.

69 Langar, K., Lorieux, M., Desmarais, E., Griveau, Y., Gentzbittel, L., and Bervillé, A. (2003) Combined mapping of DALP and AFLP markers in cultivated sunflower using F9 recombinant inbred lines. *Theor. Appl. Genet.*, **106**, 1068–1074.

70 Hu, J. and Vick, B.A. (2003) Target region amplification polymorphism: a novel marker technique for plant genotyping. *Plant Mol. Biol. Rep.*, **21**, 289–294.

71 Hu, J., Chen, J., Berville, A., and Vick, B.A. (2004) High potential of TRAP markers in sunflower genome mapping. Proceedings of the 16th International Sunflower Conference 29 Aug.–2 Sept., Fargo, ND, USA, pp. 665–671.

72 Hu, J. (2006) Defining the sunflower (*Helianthus annuus* L.) linkage group ends with the *Arabidopsis*-type telomere sequence repeat-derived markers. *Chromosome Res.*, **14**, 535–548.

73 Chen, J., Hu, J., Vick, B.A., and Jan, C.C. (2006) Molecular mapping of a nuclear male-sterility gene in sunflower (*Helianthus annuus* L.) using TRAP and SSR markers. *Theor. Appl. Genet.*, **113**, 122–127.

74 Hu, J., Chen, J., Gulya, T.J., and Miller, J.F. (2004) TRAP markers for a sunflower downy mildew resistance gene from a new *Helianthus annuus* source, PI468435. Proceedings of the 16th International Sunflower Conference Aug. 29–Sept. 2, Fargo, ND, USA, pp. 623–629.

75 Yue, B., Cai, X., Vick, B.A., and Hu, J. (2009) Genetic diversity and relationships among 177 public sunflower inbred lines assessed by TRAP markers. *Crop Sci.*, **49**, 1242–1249.

76 Yue, B., Vick, B.A., Cai, X., and Hu, J. (2010) Genetic mapping for the *Rf1* (fertility restoration) gene in sunflower (*Helianthus annuus* L.) by SSR and TRAP markers. *Plant Breed.*, **129**, 24–28.

77 León, A.J., Andrade, F.H., and Lee, M. (2003) Genetic analysis of seed oil concentration across generations and environments in sunflower (*Helianthus annuus* L.). *Crop Sci.*, **43**, 135–140.

78 León, A.J., Lee, M., and Andrade, F.H. (2001) Quantitative trait loci for growing degree days to flowering and photoperiod response in sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.*, **102**, 497–503.

79 León, A.J., Andrade, F.H., and Lee, M. (2000) Genetic mapping of factors

affecting quantitative variation for flowering in sunflower (*Helianthus annuus* L.). *Crop Sci.*, **40**, 404–407.
80 León, A.J., Lee, M., Rufener, G.K., Berry, S.T., and Mowers, R.P. (1996) Genetic mapping of a locus (*hyp*) affecting seed hypodermis color in sunflower. *Crop Sci.*, **36**, 1666–1668.
81 León, A.J., Lee, M., Rufener, G.K., Berry, S.T., and Mowers, R.P. (1995) Use of RFLP markers for genetic linkage analysis of oil percentage in sunflower seed. *Crop Sci.*, **35**, 558–564.
82 Lu, Y.H., Gagne, G., Grezes-Besset, B., and Blanchard, P. (1999) Integration of a molecular linkage group containing broomrape resistance gene *Or5* into an RFLP map in sunflower. *Genome*, **42**, 453–456.
83 Bert, P.F., Tourvieille de Labrouhe, D., Philippon, J., Mouzeyar, S., Jouan, I., Nicolas, P., and Vear, F. (2001) Identification of a second linkage group carrying genes controlling resistance to downy mildew (*Plasmopara halstedii*) in sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.*, **103**, 992–997.
84 Perez-Vich, B. (2002) Stearoyl-ACP and oleoyl-PC desaturase genes cosegregate with quantitative trait loci underlying high stearic and high oleic acid mutant phenotypes in sunflower. *Theor. Appl. Genet.*, **104**, 338–349.
85 Al-Chaarani, G.R., Roustaee, A., Gentzbittel, L., Mokrani, L., Barrault, B., Dechamp-Guillaume, G., and Sarrafi, A. (2002) A QTL analysis of sunflower partial resistance to downy mildew (*Plasmopara halstedii*) and black stem (*Phoma macdonaldii*) by the use of recombinant inbred lines (RILs). *Theor. Appl. Genet.*, **104**, 490–496.
86 Yu, J.K., Mangor, J., Thompson, L., Edwards, K.J., Slabaugh, M.B., and Knapp, S.J. (2002) Allelic diversity of simple sequence repeat markers among elite inbred lines in cultivated sunflower. *Genome*, **45**, 652–660.
87 Paniego, N., Echaide, M., Muñoz, M., Fernández, L., Torales, S., Faccio, P., Fuxan, I., Carrera, M., Zandomeni, R., Suárez, I., and Hopp, E.H. (2002) Microsatellite isolation and characterization in sunflower (*Helianthus annuus* L.). *Genome*, **45**, 34–43.
88 Tang, S., Yu, J.K., Slabaugh, M.B., Shintani, D.K., and Knapp, S.J. (2002) Simple sequence repeat map of the sunflower genome. *Theor. Appl. Genet.*, **105**, 1124–1136.
89 Tang, S., Kishore, V.K., and Knapp, S.J. (2003) PCR-multiplexes for a genome-wide framework of simple sequence repeat marker loci in cultivated sunflower. *Theor. Appl. Genet.*, **107**, 6–19.
90 Yu, J.K., Tang, S., Slabaugh, M.B., Heesacker, A., Cole, G., Herring, M., Soper, J., Han, F., Chu, W.C., Webb, D.M., Thompson, L., Edwards, K.J., Berry, S., Leon, A.J., Grondona, M., Olungu, C., Maes, N., and Knapp, S.J. (2003) Towards a saturated molecular genetic linkage map for cultivated sunflower. *Crop Sci.*, **43**, 367–387.
91 http://www.sunflower.uga.edu/cmap/ (accessed 30 March 2011).
92 http://www.ncbi.nlm.nih.gov/nucest (accessed 30 March 2011).
93 http://www.compositdb.ucdavis.edu (accessed 30 March 2011).
94 http://www.cgpdb.ucdavis.edu (accessed 30 March 2011).
95 http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=4232 (accessed 30 March 2011).
96 Lai, Z., Livingstone, K., Zou, Y., Church, S.A., Knapp, S.J., Andrews, J., and Rieseberg, L.H. (2005) Identification and mapping of SNPs from ESTs in sunflower. *Theor. Appl. Genet.*, **111** (8), 1532–1544.
97 Pashley, C.H., Ellis, J.R., McCauley, D.E., and Burke, J.M. (2006) EST databases as a source for molecular markers: lessons from *Helianthus*. *J. Hered.*, **97**, 381–388.
98 Heesacker, A., Kishore, V.K., Gao, W., Tang, S., Kolkman, J.M., Gingle, A., Matvienko, M., Kozik, A., Michelmore, R.M., Lai, Z., Riesberg, L.H., and Knapp, S.J. (2008) SSRs and INDELs mined from the sunflower EST database: abundance, polymorphisms, and cross-taxa utility. *Theor. Appl. Genet.*, **117** (7), 1021–1029.
99 Talia, P., Nishinakamasu, V., Hopp, E.H., Heinz, R.A., and Paniego, N. (2010) Genetic mapping of EST-SSRs, SSR and

InDels to improve saturation of genomic regions in a previously developed sunflower map. *Electron. J. Biotechnol.* doi: 10.2225/vol13-issue6-fulltext-14

100 Wright, S.I., Bi, X.X., Schroeder, S.G., Yamasaki, M., Doebly, J.F., McMullen, M.D., and Gaut, B.S. (2005) The effects of artificial selection on the maize genome. *Science*, **308**, 1310–1314.

101 Fusari, C., Lia, V., Nishinakamasu, V., Zubrzycki, J., Puebla, A., Maligne, A., Hopp, E., Heinz, R., and Paniego, N. (2010) Single nucleotide polymorphism genotyping by heteroduplex analysis in sunflower (*Helianthus annuus* L.). *Mol. Breed.* doi: 10.1007/s11032-010-9462-9

102 Gao, W., Gibson, R., Nipper, R., Gribbing, J., Kumpatla, S., and Greene, T. (2010) SNP discovery in sunflower using restriction-site associated DNA sequencing technology. Plant Biology 2010, July 31August 4, 2010. American Society of Biology. Abstract # P09034.

103 Kane, N., Knapp, S., Burke, J., Vincourt, P., and Rieseberg, L.H. (2010) Sequencing the genome sunflower. 18th Plant and Animal Genome Conference, January 9–13, 2010, San Diego, CA.

104 Jan, C.C. and Vick, B.A. (2005) Registration of seven cytoplasmic male-sterile and four fertility restoration sunflower germplasms. *Crop Sci.*, **48** (4), 1829–1830.

105 Roath, W.W., Miller, J.F., and Gulya, T.J. (1981) Registration of RHA801 sunflower germplasm. Reg. No. GP5 *Crop Sci.*, **21**, 479.

106 Mokrani, L., Gentzbittel, L., Azanza, F., Fitamant, L., Al-Chaarani, G., and Sarrafi, A. (2002) Mapping and analysis of quantitative trait loci for grain oil content and agronomic traits using AFLP and SSR in sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.*, **106**, 149–156.

107 Bert, P.F., Jouan, I., Tourvieille de Labrouhe, D., Serre, F., Nicolas, P., and Vear, F. (2002) Comparative genetic analysis of quantitative traits in sunflower (*Helianthus annuus* L.). 1. QTL involved in resistance to *Sclerotinia sclerotiorum* and *Diaporthe helianthi*. *Theor. Appl. Genet.*, **105**, 985–993.

108 Tang, S., Leon, A., Bridges, W.C., and Knapp, S.J. (2006) Quantitative trait loci for genetically correlated seed traits are tightly linked to branching and pericarp pigment loci in sunflower. *Crop Sci.*, **46**, 721–734.

109 Mestries, E., Gentzbittel, L., Tourvieille de Labrouhe, D., Nicolas, P., and Vear, F. (1998) Analyses of quantitative trait loci associated with resistance to *Sclerotinia sclerotiorum* in sunflowers (*Helianthus annuus* L.) using molecular markers. *Mol. Breed.*, **4**, 215–226.

110 Bert, P.F., Jouan, I., Tourvieille de Labrouhe, D., Serre, F., Philippon, J., Nicolas, P., and Vear, F. (2003) Comparative genetic analysis of quantitative traits in sunflower (*Helianthus annuus* L.). 2. Characterisation of QTL involved in developmental and agronomic traits. *Theor. Appl. Genet.*, **107**, 181–189.

111 Hajduch, M., Casteel, J.E., Tang, S., Hearne, L.B., Knapp, S.J., and Thelen, J.J. (2007) Proteomic analysis of near isogenic sunflower varieties differing in seed oil traits. *J. Proteome Res.*, **6**, 3232–3241.

112 Fonts, C., Andrade, F.H., Grondona, M., Hall, A., and León, A.J. (2008) Phenological characterization of near-isogenic sunflower families bearing two QTLs for photoperiodic response. *Crop Sci.*, **48**, 1579–1585.

113 Ebrahimi, A., Maury, P., Berger, M., Poormohammad Kiani, S., Nabipour, A., Shariati, F., Grieu, P., and Sarrafi, A. (2008) QTL mapping of seed-quality traits in sunflower recombinant inbred lines under different water regimes. *Genome*, **51**, 599–615.

114 Poormohammad Kiani, S., Grieu, P., Maury, P., Hewezi, T., Gentzbittel, L., and Sarrafi, A. (2007) Genetic variability for physiological traits under drought conditions and differential expression of water stress-associated genes in sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.*, **114** (2), 193–207.

115 Poormohammad Kiani, S., Maury, P., Nouri, L., Ykhlef, N., Grieu, P., and Sarrafi, A. (2009) QTL analysis of yield-related traits in sunflower under different water treatments. *Plant Breed.*, **4** (128), 363–373.

116 Liu, X. and Baird, Wm.V. (2003) Differential expression of genes regulated in response to drought or salinity stress in sunflower. *Crop Sci.*, **43** (2), 678–687.

117 Reynolds, M. and Tuberosa, R. (2008) Translational research impacting on crop productivity in drought-prone environments. *Curr. Opin. Plant. Biol.*, **11**, 171–179.

118 Zhang, J., Nguyen, H.T., and Blum, A. (1999) Genetic analysis of osmotic adjustment in crop plants. *J. Exp. Bot.*, **50** (332), 291–302.

119 Ali, M., Jensen, C.R., Mogensen, V.O., Andersen, M.N., and Henson, I.E. (1999) Root signalling and osmotic adjustment during intermittent soil drying sustain grain yield of field grown wheat. *Field Crops Res.*, **62**, 35–52.

120 Blum, A. (2005) Drought resistance, water-use efficiency, and yield potential: are they compatible, dissonant, or mutually exclusive? *Aust. J. Agric. Res.*, **56**, 1159–1168.

121 Chimenti, C.A. and Hall, A.J. (1993) Genetic variation and changes with ontogeny of osmotic adjustment in sunflower (*Helianthus annuus* L.). *Euphytica*, **71** (3), 201–210.

122 Chimenti, C.A., Pearson, J., and Hall, A.J. (2002) Osmotic adjustment and yield maintenance under drought in sunflower. *Field Crops Res.*, **75** (2–3), 235–246.

123 Jamaux, I., Steinmetz, A., and Belhassen, E. (1997) Looking for molecular and physiological markers of osmotic adjustment in sunflower. *New Phytol.*, **137**, 117–127.

124 Poormohammad Kiani, S., Talia, P., Maury, P., Grieu, P., Heinz, R., Perrault, A., Nishinakamasu, V., Hopp, E., Gentzbittel, L., Paniego, N., and Sarrafi, A. (2007) Genetic analysis of plant water status and osmotic adjustment in recombinant inbred lines of sunflower under two water treatments. *Plant Sci.*, **172** (4), 773–787.

125 Connor, D.J. and Jones, T.R. (1985) Response of sunflower to strategies of irrigation II. Morphological and physiological responses to water stress. *Field Crops Res.*, **12**, 91–103.

126 Cox, W.J. and Jolliff, G.D. (1986) Growth and yield of sunflwer and soybean under soil water deficits. *Agron. J.*, **78**, 226–230.

127 Guiducci, M. (1988) Effect of water deficit on leaf area development and PAR absorption of a sunflower summer crop. Proceedings of 12th International Sunflower Conference, Novi Sad, Yugoslavia, 25–29 July, 1988. International Sunflower Association, Toowoomba, Australia, pp. 89–94.

128 Sadras, V.O., Whitfield, D.M., and Connor, D.J. (1991) Transpiration efficiency in crops of semidwarf and standard-height sunflower. *Irrig. Sci.*, **12**, 87–91.

129 Sadras, V.O., Whitfield, D.M., and Connor, D.J. (1991) Regulation of evapotranspiration and its partitioning between transpiration and soil evaporation by sunflower crops. A comparison between hybrids of different stature. *Field Crops Res.*, **28**, 17–37.

130 Connor, D.J., Palta, J.A., and Jones, T.R. (1985) Response of sunflower to strategies of irrigation. III. Crop photosynthesis and transpiration. *Field Crop Res.*, **12**, 281–283.

131 Yegapan, T.M., Paton, D.M., Gates, C.T., and Muller, W.J. (1982) Water stress in sunflower (*Helianthus annus* L.). 3. Response of cypsela size. *Ann. Bot.*, **49**, 69–75.

132 Reymond, M., Muller, B., Leonardi, A., Charcosset, A., and Tardieu, F. (2003) Combining quantitative trait loci analysis and an ecophysiological model to analyze the genetic variability of the responses of maize leaf growth to temperature and water deficit. *Plant Physiol.*, **131**, 664–675.

133 Aguirrezábal, L., Bouchier-Combaud, S., Radziejwoski, A., Dauzat, M., Cookson, S.J., and Granier, G. (2006) Plasticity to soil water deficit in *Arabidopsis thaliana*: dissection of leaf development into underlying growth dynamic and cellular variables reveals invisible phenotypes. *Plant Cell Environ.*, **29**, 2216–2227.

134 Alves, A.A.C. and Setter, T.L. (2004) Response of cassava leaf area expansion to water deficit: cell proliferation, cell expansion and delayed development. *Ann. Bot.*, **94**, 605–613.

135 Pereyra-Irujo, G.A., Velázquez, L., Lechner, L., and Aguirrezábal, L.A.N. (2008) Genetic variability for leaf growth rate and duration under water deficit in sunflower: analysis of responses at cell, organ, and plant level. *J. Exp. Bot.*, **59** (8), 2221–2232.

136 Pereyra-Irujo, G., Velázquez, L., Granier, C., and Aguirrezábal, L.A.N. (2007) A method for drought tolerance screening in sunflower. *Plant Breed.*, **126**, 445–448.

137 Aguirrezabal, L.A.N., Bouchier-Combaud, S., Radziejwoski, A., Dauzat, M., Cookson, S.J., and Granier, C. (2006) Water deficit in *Arabidopsis thaliana*: dissection of leaf development into underlying growth dynamic and cellular variables reveals invisible phenotypes. *Plant Cell Environ.*, **29**, 2216–2227.

138 Cookson, S.J. and Granier, C. (2006) A dynamic analysis of the shade-induced plasticity in *Arabidopsis thaliana* rosette leaf development reveals new components of the shade-adaptive response. *Ann. Bot.*, **97**, 443–452.

139 Sala, C., Andrade, F., and Pereyra, V. (1991) *Variabilidad para la Tolerancia a las Bajas Temperaturas Durante la Germinación y la Emergencia del Girasol*, Actas 1ª Reunión Nac de Oleaginosos, Rosario, Argentina, pp. 366–371.

140 Valinoti, G., Sala, C., Andrade, F., and Pereyra, V. (1991) *Variabilidad Genotípica para la tasa de Crecimiento Relativo Durante las fases Iniciales del Crecimiento del Girasol*, Actas 1ª Reunión Nac de Oleaginosos, Rosario, Argentina, pp. 407–412.

141 Bradlow, J. (1990) Chilling sensitivity of photosynthetic oil-seedlings. I. Cotton and sunflower. *J. Exp. Bot.*, **41**, 1585–1593.

142 Allinne, C., Maury, P., Sarrafi, A., and Grieu, P. (2009) Genetic control of physiological traits associated to low temperature growth in sunflower under early sowing conditions. *Plant Sci.*, **177** (4), 349–359.

143 Thomas, H. and Smart, C.M. (1993) Crops that stay green. *Ann. Appl. Biol.*, **123**, 193–219.

144 Thomas, H. and Howarth, C.J. (2000) Five ways to stay green. *J. Exp. Bot.*, **51**, 329–337.

145 de la Vega, A.J., Cantore, M.A., Sposaro, M.M., Trápani, N., López Pereirae, M., and Hall, A.J. (2011) Canopy stay-green and yield in non-stressed sunflower. *Field Crops Res.*, **121**, 175–185.

146 Schneiter, A., Jones, J.M., and Hammond, J.J. (1987) Simulated hail research in sunflower: defoliation. *Agron. J.*, **79**, 431–434.

147 Sposaro, M.M., Chimenti, C.A., and Hall, A.J. (2008) Root lodging in sunflower, variations in anchorage strength across genotypes, soil types, crop population densities and crop developmental stages. *Field Crops Res.*, **106**, 179–186.

148 Hall, A.J., Sposaro, M.M., and Chimenti, C.A. (2010) Stem lodging in sunflower: variations in stem failure moment of force and structure across crop population densities and post-anthesis developmental stages in two genotypes of contrasting susceptibility to lodging. *Field Crops Res.*, **116**, 46–51.

149 Cukadar-Olmedo, B. and Miller, J.F. (1997) Inheritance of the stay green trait in sunflower. *Crop Sci.*, **37**, 150–153.

150 Harris, K., Subudhi, P.K., Borrell, A., Jordan, D., Rosenow, D., Nguyen, H., Klein, P., Klein, R., and Mullet, J. (2007) Sorghum stay-green QTL individually reduce post-flowering drought-induced leaf senescence. *J. Exp. Bot.*, **58** (2), 327–338.

151 Kassahun, B., Bidinger, F.R., Hash, S.T., and Kuruvinashetti, M.S. (2010) Stay-green expression in early generation sorghum [*Sorghum bicolor* (L.) Moench] QTL introgression lines. *Euphytica*, **172**, 351–362.

152 Zheng, H.J., Wu, A.Z., Zheng, C.C., Wang, Y.F., Cai, R., Shen, X.F., Xu, R.R., Liu, P., Kong, L.J., and Dong, S.T. (2009) QTL mapping of maize (*Zea mays*) stay-green traits and their relationship to yield. *Plant Breed.*, **128** (1), 54–62.

153 Ennos, A.R., Crook, M.J., and Grimshaw, C. (1993) A comparative study of the anchorage systems of Himalayan balsam *Impatiens glandulifera* and mature sunflower (*Helianthus annuus*). *J. Exp. Bot*, **44**, 133–146.

154 Sposaro, M.M., Berry, P.M., Sterling, M., Halla, A.J., and Chimenti, C.A. (2010)

Modelling root and stem lodging in sunflower. *Field Crops Res.*, **119**, 125–134.
155 López Pereira, M., Salvatelli, F., Trápani, N., and Hall, A.J. (2004) Intraspecific variability of sunflower responses to crop density. Proceedings of the 16th International Sunflower Conference, Fargo, North Dakota, USA, August 29–September 2, p. 2004.
156 Vrânceanu, A.V. (1970) Advances in sunflower breeding in Romania. Proceedings of the 4th International Sunflower Conference, Memphis, TN, 23–25 June 1970, pp. 136–148.
157 Fick, G.N. (1978) Breeding and genetics, in *Sunflower Science and Technology, Agronomy Series* (ed. J.F. Carter), ASA, CSSA and SSSA, Madison, WI, USA, pp. 279–338.
158 Beretta de Berger, A. and Miller, J.F. (1984) Genetic study of two sources of reduced height in sunflower. Proceedings of the 6th Sunflower Research Workshop, Bismarck, ND, 1 Feb. 1984. National Sunflower Association, Bismarck, ND, pp. 11–12.
159 Beretta de Berger, A. and Miller, J.F. (1985) Estudio genético de seis fuentes de estatura reducida de planta en girasol. Proceedings of the 11th International Sunflower Conference, Mar del Plata, Argentina, pp. 651–657.
160 Cecconi, F., Gaetani, M., Lenzi, C., and Durante, M. (2002) The sunflower dwarf mutant *dw1*: effects of gibberellic acid treatment. *Helia*, **25** (36), 161–166.
161 Jagadeesan, S., Kandasamy, G., Manivannan, N., and Muralidharan, V. (2008) Valuable sunflower dwarf mutant. *Helia*, **31** (49), 79–82.
162 Miller, J.F. and Hammond, J.J. (1991) Inheritance of reduced height in sunflower. *Euphytica*, **53**, 131–136.
163 Tolmachyov, V.V. (1991) Sunflower plants with reduced height, in *Sunflower Biology, Plant Breeding and Production Technology* (eds O. Tikhonov, N. Bochkarev, and A.B. Dyakiv), Agropomizdat, Moscow, Russia, pp. 44–45.
164 Miller, J.F. and Gulya, T.J. (1989) Registration of seven reduced height sunflower restorer germplasm lines. *Crop Sci.*, **29**, 1332–1333.
165 Miller, J.F. (1993) Registration of two reduced-height (HA378 and HA379) and two early-maturity (HA380 and RHA381) sunflower germplasm lines. *Crop Sci.*, **33**, 221.
166 Velasco, L., Pérez-Vich, B., Muñoz-Ruz, J., and Fernández-Martínez, J.M. (2003) Inheritance of reduced plant height in the sunflower line Dw89. *Plant Breed.*, **122**, 441–443.
167 Tomita, M. (2009) Introgression of Green Revolution *sd1* gene into isogenic genome of rice super cultivar Koshihikari to create novel semidwarf cultivar "Hikarishinseiki" (Koshihikari-sd1). *Field Crops Res.*, **114** (2), 173–181.
168 Hedden, P. (2003) The genes of the Green Revolution. *Trends Genet.*, **19** (1), 5–9.
169 Chubb, W.O. (1975) Weed competition in sunflower. Manitoba Agron. Conf. (Winnipeg, Man.) Tech Pap, pp. 119–132.
170 Robinson, R.G. (1973) The sunflower crop in Minnesota. *Minn. Agric. Ext. Bull.*, 299.
171 Bedmar, F., Leaden, M.I., and Eyherabide, J.J. (1983) Efectos de la competencia de las malezas con el girasol (*Helianthus annuus* L.). *Malezas*, **11** (4), 51–61.
172 Fleck, N.G., Pinto, J.J.O., and Mengarda, I.P. (1989) Interferencia de plantas daninhas na cultura do girassol. Competição no tempo. *Pesq. Agropec. Bras.*, **24** (9), 1139–1147.
173 Brighenti, A.M., Castro, C., Oliveira, R.S., Jr., Scapim, C.A., Voll, E., and Gazziero, D.L.P. (2004) Períodos de interferência de plantas daninhas na cultura do girasol. *Planta Daninha*, **22** (2), 251–257.
174 Blamey, F.P.C., Edwards, D.G., and Asher, C.J. (1987) *Nutritional Disorders of Sunflower*, Depatment of Agriculture, University of Queensland, St. Lucia, Australia.
175 Gillespie, G.R. and Miller, S.D. (1980) Differential response of sunflower to herbicides. *Proc. North Central Weed Control Conf.*, **35**, 31–35.
176 Gillespie, G.R. and Miller, S.D. (1983) Sunflower (*Helianthus annuus*) response to diclofop. *Weed Sci.*, **31** (4), 500–503.
177 Kaspar, M., Grondona, M., Leon, A., and Zambelli, A. (2010) Selection of a

sunflower line with multiple herbicide tolerance that is reversed by the P450 inhibitor Malathion. *Weed Sci.*, **59** (2), 232–237.

178 Yuan, J.S., Tranel, P.J., and Stewart, N., Jr. (2006) Non-target site herbicide resistance: a family business. *Trends Plant Sci.*, **12** (1), 6–13.

179 Batard, Y., LeRet, M., Schalk, M., Robineau, T., Durst, F., and Werck-Reichhart, D. (1988) Molecular cloning and functional expression in yeast of CYP76B1, a xenobiotic-inducible 7-ethoxycoumarin *O*-de-ethylase from *Helianthus tuberosus*. *Plant J.*, **14** (1), 111–120.

180 Cabello-Hurtado, F., Batard, Y., Salaün, J.P., Durst, F., Pinot, F., and Werck-Reichhart, D. (1998) Cloning, expression in yeast, and functional characterization of CYP81B1, a plant P450 which catalyzes in-chain hydroxylation of fatty acids. *J. Biol. Chem.*, **273**, 7260–7267.

181 Robineau, T., Batard, Y., Nedelkina, S., Cabello-Hurtado, F., LeRet, M., Sorokine, O., Didierjean, L., and Werck-Reichhart, D. (1988) The chemically inducible plant cytochrome P450 CYP76B1 actively metabolizes phenylureas and other xenobiotics. *Plant Physiol.*, **118** (3), 1049–1056.

182 Brown, H.M. (1990) Mode of action, crop selectivity, and soil relations of the sulfonylurea herbicides. *Pesticide Sci.*, **29** (3), 263–281.

183 Tan, S., Evans, R.R., Dahmer, M.L., Singh, B.K., and Shaner, D.L. (2005) Imidazolinonetolerant crops: history, current status and future. *Pest Manag. Sci.*, **61**, 246–257.

184 Shaner, D.L., Anderson, P.C., and Stidham, M.A. (1984) Imidazolinones: potent inhibitors of acetohydroxyacid synthase. *Plant Physiol.*, **76**, 545–546.

185 Ray, T.B. (1984) Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiol*, **75**, 827–831.

186 Singh, B.K. (1999) Biosynthesis of valine, leucine and isoleucine, in *Plant Aminoacids* (ed. B.K. Singh), Marcel Dekker Inc., New York, pp. 227–247.

187 Subramanian, M.V. and Gerwick, B.C. (1989) Inhibition of acetolactate synthase by triazolopyrimidines: a review of recent developments, in *Biocatalysis in Agricultural Biotechnology* (eds J.R. Whitaker and P.E. Sonnet), American Chemical Society, Washington, DC, pp. 277–288.

188 Subramanian, M.V., Hung, H.Y., Dias, J.M., Miner, V.W., Butler, J.H., and Jachetta, J.J. (1990) Properties of mutant acetolactate synthases resistant to triazolopyrimidine sulfonanilide. *Plant Physiol.*, **94**, 239–244.

189 Santel, H.J., Bowden, B.A., Sorenson, V.M., Mueller, K.H., and Reynolds, J. (1999) Flucarbazone-sodium: a new herbicide for grass control in wheat. *Proc. West Soc. Weed Sci.*, **52**, 124–125.

190 Tan, S., Evans, R., and Singh, B. (2006) Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. *Amino Acids*, **30** (2), 195–204.

191 Preston, C. and Mallory-Smith, C.A. (2001) Biochemical mechanisms, inheritance, and molecular genetics of herbicide resistance in weeds, in *Herbicide Resistance and World Grains* (eds S.B. Powles and D.L. Shaner), CRC Press, Boca Raton, FL, pp. 23–60.

192 Tranel, P.J. and Wright, T.R. (2002) Resistance of weeds to AHAS inhibiting herbicides: what have we learned? *Weed Sci.*, **50**, 700–712.

193 Kolkman, J.M., Slabaugh, M.B., Bruniard, J.M., Berry, S., Bushman, B.S., Olungu, C., Maes, N., Abratti, G., Zambelli, A., Miller, J.F., Leon, A., and Knapp, S.J. (2004) Acetohydroxyacid synthase mutations conferring resistance to imidazolinone or sulfonylurea herbicides in sunflower. *Theor. Appl. Genet.*, **109**, 1147–1159.

194 Bruniard, J.M. and Miller, J.F. (2001) Inheritance of imidazolinone herbicide resistance in sunflower. *Helia*, **24**, 11–16.

195 Sala, C.A., Bulos, M., Echarte, A.M., Whitt, S.R., and Ascenzi, R. (2008) Molecular and biochemical characterization of an induced mutation conferring imidazolinone resistance in sunflower. *Theor. Appl. Genet.*, **108**, 115–112.

196 Al-Khatib, K., Baumgartner, J.R., Peterson, D.E., and Currie, R.S. (1998) Imazethapyr resistance in common sunflower (*Helianthus annuus*). *Weed Sci.*, **46**, 403–407.

197 Miller, J.F. and Al-Khatib, K. (2002) Registration of imidazolinone herbicide-resistant sunflower maintainer (HA425) and fertility restorer (RHA426 and RHA427) germplasms. *Crop Sci.*, **42**, 988–989.

198 Sala, C.A., Bulos, M., and Echarte, A.M. (2008) Genetic analysis of an induced mutation conferring imidazolinone resistance in sunflower. *Crop Sci.*, **48**, 1817–1822.

199 Sala, C.A., Bulos, M., Echarte, A.M., Whitt, S., Budziszewski, G., Howie, W., Singh, B., and Weston, B. (2008) Development of CLHA-Plus: a novel herbicide tolerance trait in sunflower conferring superior imidazolinone tolerance and ease of breeding. Proceedings of the 17th International Sunflower Conference, Córdoba, España, pp. 489–494.

200 Sala, C.A., Bulos, M., Altieri E., and Weston B. (2011) Response to imazapyr and dominance relationships of two imidazolinone-tolerant alleles at the Ahasl1 locus of sunflower. *Theor. Appl. Genet.* (in press) doi: 10.1007/s00122-011-1713-6

201 Al-Khatib, K., Baumgartner, J.R., and Currie, R.S. (1999) Survey of common sunflower (*Helianthus annuus*) resistance to ALS-inhibiting herbicides in northeast Kansas. Proceedings of the 21th Sunflower Research Workshop, National Sunflower Association, Bismark, ND, pp. 210–215.

202 Miller, J.F. and Al-Khatib, K. (2004) Registration of two oilseed sunflower genetic stocks, SURES-1 and SURES-2, resistant to tribenuron herbicide. *Crop Sci.*, **44**, 1037–1038.

203 Gabard, J.M. (2004) Sulfonylurea-tolerant sunflower plants. United States Patent Application 20050044587 filed 2004.

204 Decision Document DD2008-69 (2008) Determination of the Safety of Pioneer Hi-Bred Production Ltd.'s Sulfonylurea-Tolerant ExpressSun™ Sunflower (*Helianthus annuus* L.) SU7. Canadian Food Inspection Agency (http://www.inspection.gc.ca/english/plaveg/bio/dd/dd0869e.shtml).

205 Sala, C.A., Bulos, M., Echarte, A.M., Singh, B.K., Weston, B.J., and Whitt, S.R. (2008) Herbicide-resistant sunflower plants with multiple herbicide resistant alleles of *AHASL1* and methods of use. WO Patent application PCT/US2008/059125, filed April 2, 2008.

206 Sala, C.A. and Bulos, M. (2011) Inheritance and molecular characterization of broad range tolerance to herbicides targeting acetohydroxyacid synthase in sunflower. *Theor. Appl. Genet.* (in press) doi: 10.1007/s00122-011-1710–9

207 Dure, L., III, Crouch, M., Harada, J., Ho, T.-H.D., Mundy, J., Quatrano, R., Thomas, T., and Sung, Z.R. (1989) Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Mol. Biol.*, **12**, 475–486.

208 Close, T.J. (1997) Dehydrins: a commonality in the response of plants to dehydration and low temperature. *Physiol. Plant*, **100**, 291–296.

209 Close, T.J. (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiol. Plant*, **97**, 795–803.

210 Mouillon, J.M., Eriksson, S.K., and Harryson, P. (2008) Mimicking the plant cell interior under water stress by macromolecular crowding: disordered dehydrin proteins are highly resistant to structural collapse. *Plant Physiol.*, **148**, 1925–1937.

211 Galvez, A.F., Gulick, P.J., and Dvorak, J. (1993) Characterization of the early stages of genetic salt-stress responses in salt-tolerant *Lophopyrum elongatum*, salt-sensitive wheat, and their amphiploid. *Plant Physiol.*, **103**, 257–265.

212 Moons, A., Bauw, G., Prinsen, E., Van Montagu, M., and Van Der Straeten, D. (1995) Molecular and physiological responses to abscisic acid and salts in roots of salt-sensitive and salt-tolerant indica rice varieties. *Plant Physiol.*, **107**, 177–186.

213 Arora, R. and Wisniewski, M.E. (1994) Cold acclimation in genetically related (sibling) deciduous and evergreen peach

(*Prunus persica* L. Batsch). II. A 60-kilodalton bark protein in cold-acclimated tissues of peach is heat stable and related to the dehydrin family of proteins. *Plant Physiol.*, **105**, 95–101.

214 Danyluk, J., Houde, M., Rassart, E., and Sarhan, F. (1994) Differential expression of a gene encoding an acidic dehydrin in chilling sensitive and freezing tolerant Gramineae species. *FEBS Lett.*, **344**, 20–24.

215 Artlip, T.S., Callahan, A.M., Bassett, C.L., and Wisniewski, M.E. (1997) Seasonal expression of a dehydrin gene in sibling deciduous and evergreen genotypes of peach (*Prunus persica* L. Batsch). *Plant Mol. Biol.*, **33**, 61–70.

216 Cellier, F., Conéjéro, G., Breitler, J.-C., and Casse, F. (1998) Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. *Plant Physiol.*, **116**, 319–328.

217 Ouvrard, O., Cellier, F., Ferrare, K., Tousch, D., Lamaze, T., Dupuis, J.-M., and Casse-Delbart, F. (1996) Identification and expression of water stress- and abscisic acid-regulated genes in a drought tolerant sunflower genotype. *Plant Mol. Biol.*, **31**, 819–829.

218 Natali, L., Giordani, T., and Cavallini, A. (2003) Sequence variability of a dehydrin gene within *Helianthus annuus*. *Theor. Appl. Genet.*, **106**, 811–818.

219 Giordani, T., Buti, M., Natali, L., Pugliesi, C., Cattonaro, F., Morgante, M., and Cavallini, A. (2011) An analysis of sequence variability in eight genes putatively involved in drought response in sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.*, **122**, 1039–1049.

220 Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. *Curr. Opin. Biotechnol.*, **17**, 113–22.

221 Valliyodan, B. and Nguyen, H.T. (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Curr. Opin. Plant. Biol.*, **9**, 189–95.

222 Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 571–99.

223 Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **53**, 247–73.

224 Hazen, S.P., Wu, Y., and Kreps, J.A. (2003) Gene expression profiling of plant responses to abiotic stress. *Funct. Integr. Genomics*, **3**, 105–11.

225 Seki, M., Kamei, A., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Curr. Opin. Biotechnol.*, **14**, 194–9.

226 Wang, W., Vinocur, B., and Altman, A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, **218**, 1–14.

227 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant. Biol.*, **57**, 781–803.

228 Tran, L.-S.P., Nakashima, K., Shinozaki, K., and Yamaguchi- Shinozaki, K. (2007) Plant gene networks in osmotic stress response: from genes to regulatory networks. *Methods Enzymol.*, **428**, 109–28.

229 Chen, W. and Zhu, T. (2004) Networks of transcription factors with roles in environmental stress response. *Trends Plant Sci.*, **9**, 591–596.

230 Nakashima, K., Ito, Y., and Yamaguchi-Shinozaki, K. (2009) Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. *Plant Physiol.*, **149**, 88–95.

231 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2005) Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci.*, **10**, 88–94.

232 Udvardi, M.K., Kakar, K., Wandrey, M., Montanari, O., Murray, J., and Andriankaja, A. (2007) Legume transcription actors: global regulators of plant development and response to the environment. *Plant Physiol.*, **144**, 538–49.

233 Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L.,

Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., and Yu, G. (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science*, **290**, 2105–2110.

234 Goff, S.A., Ricke, D., Lan, T.H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B.M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W.L., Chen, L., Cooper, B., Park, S., Wood, T.C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zharkikh, A., Shen, R., Sahasrabudhe, S., Tomas, A., Cannings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R.M., Bhatnagar, S., Adey, N., Rubano, T., Tusneem, N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A., and Briggs, S. (2002) A draft sequence of the rice genome (*Oryza sativa* L. spp. *japonica*). *Science*, **296**, 79–92.

235 Riechmann, J.L. and Ratcliffe, O.J. (2000) A genomic perspective on plant transcription factors. *Curr. Opin. Plant. Biol.*, **3**, 423–434.

236 Dezar, C.A., Fedrigo, G.V., and Chan, R.L. (2005) The promoter of the sunflower HD-Zip protein gene HAHB4 directs tissue-specific expression and is inducible by water stress, high salt concentrations and ABA. *Plant Sci.*, **169**, 447–459.

237 Chan, R.L., Gonzales, D., Dezar, C.A., and Rueda, E.C. (2006) *Helianthus annuus* Hahb-10 gene coding sequence, method for generating plants with shortened life cycle and a high tolerance to herbicidal compounds and transgenic plants with that sequence. US Patent 7.674.954 B2, filed October 6, 2006 and issued March 9, 2010.

238 Manavella, P.A., Arce, A.L., Dezar, C.A., Bitton, F., Renou, J.P., Crespi, M., and Chan, R.L. (2006) Cross-talk between ethylene and drought signaling pathways is mediated by the sunflower Hahb-4 transcription factor. *Plant J.*, **48**, 125–137.

239 Gago, G.M., Almoguera, C., Jordano, J., Gonzalez, D.H., and Chan, R.L. (2002) HAHB-4, a homeobox-leucine zipper gene potentially involved in ABA-dependent responses to water stress in sunflower. *Plant Cell Environ.*, **25**, 633–640.

240 Himmelbach, A., Hoffmann, T., Leube, M., Höhener, B., and Grill, E. (2002) Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in *Arabidopsis*. *EMBO J.*, **21**, 3029–3038.

241 Mayda, E., Tornero, P., Conejero, V., and Vera, P. (1999) A tomato homeobox gene (HD-Zip) is involved in limiting the spread of programmed cell death. *Plant J.*, **20**, 591–600.

242 Meijer, A.H., de Kam, R.J., d'Erfurth, I., Shen, W., and Hoge, J.H.C. (2000) HD-Zip proteins of families I and II from rice: interactions and functional properties. *Mol. Gen. Genet.*, **263**, 12–21.

243 Olsson, A., Engström, P., and Söderman, E. (2004) The homeobox genes ATHB12 and ATHB7 encode potential regulators of growth in response to water deficit in *Arabidopsis*. *Plant Mol. Biol.*, **55**, 663–677.

244 Rueda, E.C., Dezar, C.A., Gonzalez, D.H., and Chan, R.L. (2005) HAHB-10, a sunflower homeobox-leucine zipper gene, is involved in the response to dark/light conditions and promotes a reduction of the life cycle when expressed in *Arabidopsis*. *Plant Cell Physiol.*, **46**, 1954–1963.

245 Sessa, G., Carabelli, M., Sassi, M., Ciolfi, A., Possenti, M., Mittempergher, F., Becker, J., Morelli, G., and Ruberti, I. (2005) A dynamic balance between gene activation and repression regulates the shade avoidance response in *Arabidopsis*. *Genes Dev.*, **19**, 2811–2815.

246 Morelli, G. and Ruberti, I. (2002) Light and shade in the photocontrol of *Arabidopsis* growth. *Trends Plant Sci.*, **7**, 399–404.

247 Morelli, G. and Ruberti, I. (2000) Shade avoidance responses. Driving auxin along lateral routes. *Plant Physiol.*, **122**, 621–626.

248 Chan, R.L., Gonzales, D., Dezar, C.A., and Gago, G.M. (2010) Transcription factor gene induced by water deficit conditions and abscisic acid from

Helianthus annuus, promoter and transgenic plants. US Patent application 12/690,385, filed January 20, 2010.

249 Chan, R.L. (2009) The use of sunflower transcription factors as biotechnological tools to improve yield and stress tolerance in crops. *Phyton*, **78**, 5–10.

250 Roche, J., Hewezi, T., Bouniols, A., and Gentzbittel, L. (2007) Transcriptional profiles of primary metabolism and signal transduction-related genes in response to water stress in field-grown sunflower genotypes using a thematic cDNA microarray. *Planta*, **226** (3), 601–617.

251 Collard, B.C.Y. and Mackill, D.J. (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Phil. Trans. R. Soc. B*, **363**, 557–572.

252 Sala, C., Bulos, M., Fresco, A., and Altieri, E. (2010) Marcadores moleculares y mejoramiento genético de cultivos, in *Biotecnología y Mejoramiento Vegetal II* (eds G. Levitus, V. Echenique, C. Rubinstein, E. Hopp, and L. Mroginski), Ediciones Instituto Nacional de tecnología Agropecuaria., pp. 325–338.

253 Price, A.H. (2006) Believe it or not, QTLs are accurate!. *Trends Plant Sci.*, **11**, 213–216.

254 Melchinger, A.E., Utz, H.F., and Schön, C.C. (1998) Quantitative trait locus (QTL) mapping using different testers and independent population samples in maize reveals low power of QTL detection and large bias in estimates of QTL effects. *Genetics*, **149**, 383–403.

255 Langridge, P. and Chalmers, K. (2005) The principle: identification and application of molecular markers, in *Biotechnology in Agriculture and Forestry. Molecular Marker Systems*, vol. 55 (eds H. Lorz and G. Wenzel), Springer, Berlin, Germany, pp. 3–22.

256 Xu, Y. and Crouch, J.H. (2008) Marker-assisted selection in plant breeding: from publications to practice. *Crop Sci.*, **48**, 391–407.

257 Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A., Francia, E., Marè, C., Tondelli, A., and Stanca, M. (2008) Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Res.*, **105**, 1–14.

258 Collins, N.C., Tardieu, T., and Tuberosa, R. (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? *Plant Physiol.*, **147**, 469–486.

259 Wade, M.J. (2002) A gene's eye view of epistasis, selection and speciation. *J. Evol. Biol.*, **15**, 337–346.

260 Podlich, D.W., Winkler, C.R., and Cooper, M. (2004) Mapping as you go: an effective approach for marker-assisted selection of complex traits. *Crop Sci.*, **44**, 1560–1571.

261 León, A.J., Berry, S.T., Rufener, G.K., and Mowers, R.P. (1993) Oil producing sunflowers and production thereof. US Patent 5,476,524, filed December 9, 1993 and issued December 19, 1995.

262 Lu, G., Hu, X., and Bidney, D.L. (2007) Biotechnology in agriculture and forestry, in *Transgenic Crops VI*, vol. 61 (eds E.C. Pua and M.R. Davey), Springer, Berlin, pp. 39–58.

263 Bidney, D.L. and Scelonge, C.J. (1997) Sunflower biotechnology, in *Sunflower Technology and Production, Agronomy Series* (ed. A.A. Schneiter), ASA, CSSA and SSSA, Madison, WI, USA, pp. 765–808.

264 Deglene, L., Lesignes, P., Alibert, G., and Sarrafi, A. (1997) Genetic control of organogenesis in cotyledons of sunflower (*Helianthus annuus*). *Plant Cell Tissue Organ Cult.*, **48**, 127–130.

265 Flores Berrios, E., Gentzbittel, L., Kayyal, H., and Alibert, G. (2000) AFLP mapping of QTLs for *in vitro* organogenesis traits using recombinant inbred lines in sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.*, **101**, 1299–1306.

266 Sarrafi, A. and Gentzbittel, L. (2005) Genomics as efficient tools: example sunflower breeding, in *Biotechnology in Agriculture and Forestry, Vol. 55 Molecular Marker Systems* (eds H. Lörz and G. Wenzel), Springer, Berlin, pp. 107–119.

267 Knittel, N., Gruber, V., Hahne, G., and Lénéé, P. (1994) Transformation of sunflower (*Helianthus annuus* L.): a reliable protocol. *Plant Cell Rep.*, **14** (2–3), 81–86.

268 Burrus, M., Molinier, J., Himber, C., Hunold, R., Bronner, R., Rousselin, P.,

and Hahne, G. (1996) *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.) shoot apices: transformation patterns. *Mol. Breed.*, **2** (4), 329–338.

269 Rao, K.S. and Rohini, V.K. (1999) *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): a simple protocol. *Ann. Bot.*, **83**, 347–354.

270 Lucas, O., Kallerhoff, J., and Alibert, G. (2000) Production of stable transgenic sunflower (*Helianthus annuus* L.) from wounded immature embryos by particle bombardment and co-cultivation with *Agrobacterium tumefaciens*. *Mol. Breed.*, **6** (5), 479–487.

271 Weber, S., Friedt, W., Landes, N., Molinier, J., Himber, C., Rousselin, P., Hahne, G., and Horn, R. (2003) Improved *Agrobacterium* -mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. *Plant Cell Rep.*, **21** (5), 475–482.

272 Grayburn, W.S. and Vick, B.A. (1995) Transformation of sunflower (*Helianthus annuus* L.) following wounding with glass beads. *Plant Cell Rep.*, **14** (5), 285–289.

273 Alibert, B., Lucas, O., Le Gall, V., Kallerhoff, J., and Alibert, G. (1999) Pectolytic enzyme treatment of sunflower explants prior to wounding and cocultivation with *Agrobacterium tumefaciens*, enhances efficiency of transient beta glucuronidase expression. *Physiol. Plant*, **106**, 232–237.

274 Bidney, D.L., Scelonge, C.J., Martich, J., Burrus, M., Sims, L., and Huffman, G. (1992) Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. *Plant Mol. Biol.*, **18**, 301–313.

275 Malone-Schoneberg, J.B., Scelonge, C.J., Burrus, M., and Bidney, D.L. (1994) Stable transformation of sunflower using *Agrobacterium* and split embryonic axis explants. *Plant Sci.*, **103**, 199–207.

276 Müller, A., Markus, I., and Hess, D. (2001) Stable transformation of sunflower (*Helianthus annuus* L.) using a non-meristematic regeneration protocol and green fluorescent protein as a vital marker. *Transgen. Res.*, **10**, 435–444.

277 Hewezi, T., Perrault, A., Alibert, G., and Kallerhoff, J. (2002) Dehydrating immature embryo split apices and rehydrating with *Agrobacterium tumefaciens*: a new method for genetically transforming recalcitrant sunflower. *Plant Mol. Biol. Rep.*, **20**, 335–345.

278 Lewi, D. (2004) Transformación genética del girasol. PhD Thesis, Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina.

279 Radonic, L.M., Zimmermann, J.M., Zavallo, D., López, N., and López Bilbao, M. (2006) Introduction of antifungal genes in sunflower via *Agrobacterium*. *Electron. J. Biotechnol.*, **9** (3). doi: 10.2225/vol11-issue5-fulltext-2

280 Mohamed, S., Binsfeld, P.C., Cerboncini, C., and Schnabl, H. (2003) Regeneration systems at high frequency from high oleic *Helianthus annuus* L. genotypes. *J. Appl. Bot.*, **77**, 85–89.

281 Mohamed, S., Boehm, R., and Schnabl, H. (2006) Stable genetic transformation of high oleic *Helianthus annuus* L. genotypes with high efficiency. *Plant Sci.*, **171**, 546–554.

282 Manavella, P.A. and Chan, R.L. (2009) Transient transformation of sunflower leaf discs via an *Agrobacterium*-mediated method: applications for gene expression and silencing studies. *Nat. Protoc.*, **4**, 1699–1707.

283 Cantamutto, M. and Poverene, M. (2007) Genetically modified sunflower release: opportunities and risks. *Field Crops Res.*, **101**, 133–144.

284 Rousselin, P., Molinier, J., Himber, C., Schontz, D., Prieto-Dapena, P., Jordano, J., Martini, N., Weber, S., Horn, R., Ganssmann, M., Grison, R., Pagniez, M., Toppan, A., Friedt, W., and Hahne, G. (2002) Modification of sunflower oil quality by seed-specific expression of a heterologous delta-9 stearoyl-(acyl carrier protein) desaturase gene. *Plant Breed.*, **121**, 108–116.

285 Sawahel, W. and Hagran, A. (2006) Generation of white mold disease-resistant sunflower plants expressing human lysozyme gene. *Biol. Plant*, **50**, 683–687.

286 Bervillé, A., Muller, M.H., Poinso, B., and Serieys, H. (2005) Crop ferality and

volunteerism: a threat to food security in the transgenic era? in *Ferality: Risks of Gene Flow Between Sunflower and other Helianthus Species* (ed. J. Gressel), CRC Press, Boca Raton, USA, pp. 209–229.

287 Daniell, H. (1999) Environmentally friendly approaches to genetic engineering. *In Vitro Cell. Dev. Biol. Plant*, **35**, 361–368.

288 Faure, N., Serieys, H., Berville, A., Cazaux, E., and Kaan, F. (2002) Occurrence of partial hybrids in wide crosses between sunflower (*Helianthus annuus*) and perennial species *H. mollis* and *H.orgyalis*. *Theor. Appl. Genet.*, **104**, 652–660.

289 Snow, A.A., Pilson, D., Rieseberg, L.H., Paulsen, M.J., Pleskac, N., Reagon, M.R., and Wolf, D.E. (2003) A Bt transgene reduces herbivory and enhances fecundity in wild sunflowers. *Ecol. Appl.*, **13**, 279–286.

290 Massinga, R.A., Al-KhatibK, St., Amand, P., and Miller, J.F. (2003) Gene flow from imidazolinone-resistant domesticated sunflower to wild relatives. *Weed Sci.*, **51**, 854–862.

291 Didierjean, L., Gondet, L., Perkins, R., Lau, S.-M.C., Schaller, H., O'Keefe, D.P., and Werck-Reichhart, D. (2002) Engineering herbicide metabolism in tobacco and *Arabidopsis* with CYP76B1, a cytochrome P450 enzyme from Jerusalem artichoke. *Plant Physiol.*, **130**, 179–189.

292 Vear, F. (2011) Helianthus, in *Wild Crop Relatives: Genomic and Breeding Resources, Oilseeds* (ed. C. Kole), Springer, Berlin, pp. 161–170.

293 Yin, X., Struik, P.C., and Kropff, M.J. (2004) Role of crop physiology in predicting gene-to-phenotype relationships. *Trends Plant Sci.*, **9** (9), 426–432.

294 Wollenweber, B., Porter, J.R., and Lubberstedt, T. (2005) Need for multidisciplinary research towards a second Green Revolution. *Curr. Opin. Plant. Biol.*, **8** (3), 337–341.

295 Tardieu, F. and Tuberosa, R. (2010) Dissection and modelling of abiotic stress tolerance in plants. *Curr. Opin. Plant. Biol.*, **13** (2), 206–212.

296 Lecoeur, J., Poiré-Lassus, R., Christophe, A., Pallas, B., Casadebaig, P., Debaeke, P., Vear, F., and Guilioni, L. (2011) Quantifying physiological determinants of genetic variation for yield potential in sunflower. SUNFLO: a model-based analysis. *Funct. Plant Biol.*, **38**, 246–259.

297 Salvi, S. and Tuberosa, R. (2005) To clone or not to clone plant QTLs: present and future challenges. *Trends Plant Sci.*, **10**, 297–304.

298 Sistema Integrado de Información Agropecuaria. Ministerio de Agricultura, Ganadería y Pesca de la República Argentina. Available at http://www.siia.gov.ar (accessed 30 March 2011).

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Sesame: Overcoming the Abiotic Stresses in the Queen of Oilseed Crops

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Sesame, one of the important oilseed crops, is valued for its high quality oil rich in polyunsaturated fatty acids (PUFA) and thus offers excellent health benefits. The oil also has unique antioxidative property that increases its keeping quality by preventing oxidative rancidity. However, the research efforts for developing improved sesame cultivars having tolerance to biotic and abiotic stresses have been rather meager so far. Sesame is largely cultivated in marginal lands by resource-poor farmers and is thereby prone to several abiotic stresses. The crop possesses effective tolerance to drought due to its extensive root system. Preliminary studies have been carried out in sesame regarding salt, drought, and heavy metal stress. Parameters such as root and shoot morphology, cuticle thickness, antioxidative enzymes, malondialdehyde, proline content, and so on have been assessed under stressed and control conditions. Role of stress-associated genes and their products such as lipid transfer proteins, caleosins, steroid dehydrogenase, phytostatins, γ-aminobutyric acid, metallothioneins involved in diverse stresses are under investigation. The presence of phenylpropanoid compounds, namely, lignans, an innate nonenzymatic antioxidant defense mechanism against reactive oxygen species in sesame, is a special area being researched. However, the areas that still remain untouched include waterlogging and chilling stress, both of which are highly detrimental to the crop survival. Inspite of huge repertoire of germplasm collection, limited research efforts on the use of conventional and biotechnological methodologies have resulted in minimal success in developing abiotic stress-tolerant cultivars. The absence of efficient *in vitro* regeneration protocols further compounds challenges for development of desired novel genotypes. The possible strategies that could be helpful in incorporating abiotic stress tolerance in plants have been discussed here along with the fundamental studies dealing with different stresses and their effects on sesame, followed by information on stress-related genes under focus in sesame.

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48.1
Introduction

Global population has increased with the unprecedented rate over the past few decades resulting in shortage of resources at all fronts. However, the biggest problem is the shortage of food particularly in the underdeveloped parts of the world. Providing additional land for cultivation is already becoming unrealistic due to increasing demands of urbanization on the one hand and the need to maintain and restore the much reduced forest cover on the other hand. In addition, a series of natural calamities and global environmental changes have compounded the abiotic and biotic stresses that adversely affect the productivity of the crop plants. Abiotic stress is, in fact, a general term referring to physical stresses experienced by the plant vis-a-vis the unfavorable conditions posed by the environmental factors or the substratum where the plant is growing. The negative influences of abiotic stresses such as drought, salinity, cold, chilling, flooding, and so on affect survival, biomass production and accumulation, and grain yield in most crops [1]. About 85% of crop productivity losses are due to different kinds of abiotic stresses [2], which is much more significant in comparison to the losses that occur due to insects/pests, weeds, and diseases [3].

Plants have evolved several adaptations to survive the harsh environmental conditions due to innate plasticity in their physiological and metabolic processes. The conquest of nearly the entire planet with the living organisms spanning extreme conditions on both sides of all the physical parameters provides evidence for the range of functional capability of the biological machinery and hope for the incorporation of such traits in the desired taxa. The need for the addition of traits imparting tolerance to abiotic stresses cannot be overemphasized. Both conventional breeding and biotechnological tools, individually or in combination, have yielded superior genotypes in major crop plants. However, several other crop plants that not only contribute significantly to the food and nutritional requirement but also play an important role in diversification of the crops are far from researched. Sesame (*Sesamum indicum* L.) is one such crop that deserves urgent and immediate attention of the scientific community.

48.2
Sesame: an Oilseed Crop

Sesame, an important oil yielding plant, is one of the most ancient crops known and used as oilseed. The high regard it enjoys among the users has earned it the poetic label "queen of oilseeds" [4]. Ironically, it has also been considered an "orphan crop" due to lack of research efforts ascribed to the fact that it is not a mandate crop for any international crop research institute [5]. Though sesame is cultivated on a worldwide basis for its seeds, oil, and protein, it is predominantly an annual crop of warmer areas, particularly Asia and Africa [6] where it is used both as a leafy vegetable and an oilseed crop. The presence of unique antioxidant compounds such as sesamin,

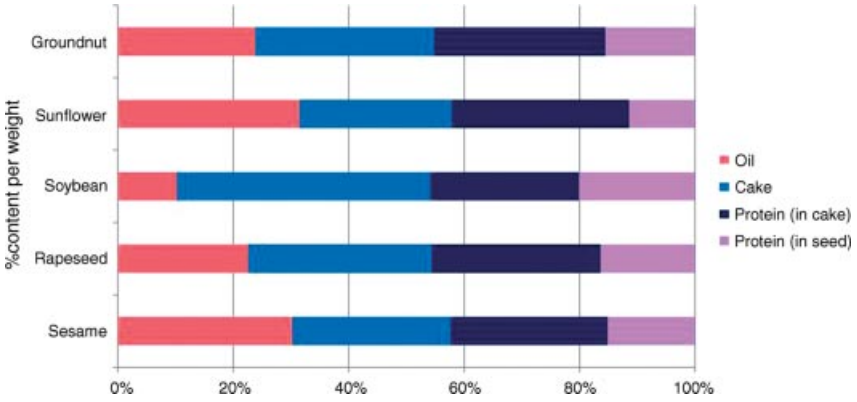

Figure 48.1 Oil and protein content of selected oilseeds (% content per weight basis) (http://www.fao.org/inpho/content/compend/text/ch05-01.htm).

sesamolin, and sesaminol makes the sesame oil one of the most stable vegetable oils in the world. The oil content ranges from 32.5 to 58.8%, which is generally greater in white than black seeds [7]. In general, the oil content compares well with other oilseed crops (Figure 48.1). The oil is rich in linoleic acid (LA) (Figure 48.2) and has been recommended for healthy diets with low LA and high alpha linoleic acid in combination with canola or mustard oil [8]. Seeds as a whole serve as nutritious food for humans and are widely used in bakery and confectionery products [9]. Sesame meal contains 35–50% protein, which is rich in tryptophan and methionine, and is used as poultry feed. Its leaves used as a vegetable are a rich source of carotene, ascorbic acid, iron, and calcium along with adequate quantities of protein [10].

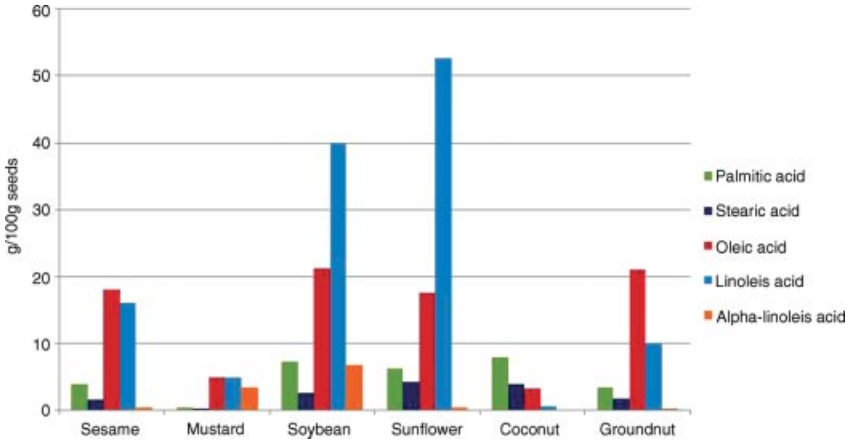

Figure 48.2 Fatty acid composition of common oilseeds. Adapted from [11, 12].

Sesame cultivation has many advantages as the seed set and yields are relatively well under high temperature and the crop can be grown even on residual moisture without any extra rainfall. Sesame crop also improves water percolation of the soil as its extensive branching system of feeder roots penetrates very deep into the soil. It can be grown in pure stands and also as a companion crop [5]. Furthermore, it responds well to additional inputs in terms of irrigation and fertilization (by at least doubling the yield capacity), and thus has an important role in intensive management systems, including sequential multiple cropping [13].

Sesame is grown in the tropical to temperate zones from about 40°N latitude to 40°S latitude [14]. The plant is adapted to many soil types, but it thrives best on well-drained, fertile soils of medium texture (typical sandy loams) and at neutral pH. In India, sesame as a sole crop is mainly cultivated in drier parts of Maharashtra, Madhya Pradesh, Rajasthan, Gujarat, Andhra Pradesh, Uttar Pradesh, and Karnataka. However, it is grown in almost all states of India either as a mixed crop or in other forms of small-scale cultivation [15]. In Northern India, the crop is taken as rain-fed *kharif* crop and in Central India as a semi-*rabi* crop. However, in the South the crop is taken in both the seasons and in the Northeast the crop is taken three times in a year.

According to the Food and Agriculture Organization of the United Nations (FAO, 2002), sesame ranks 6th in the world production as an edible oil seed (2 893 114 million ton) and 12th in the overall world production of vegetable oil (754 159 million ton). The world production of sesame seed and seed oil is 3.2 million ton and 0.8 million ton, respectively (http://www.agmrc.org/agmrc/commodity/grainoilseeds/sesame/sesameprofile.htm). However, the world production fluctuates due to local economic crop production pressures and weather conditions. India accounted for 7.4% of the world's edible oil consumption with an estimated production of 28.21 million ton of nine cultivated oilseeds in 2007–2008 (http://fcamin.nic.in/dfpd/EventDetails.asp?EventId=561&Section=Edible + Oil&ParentID=0&Parent=1&check=0). The major oilseeds responsible are groundnut, soybean, and rape/mustard seeds covering 80% of the total oilseeds grown in India. The consumption of edible oils in India reached 142.62 lakh ton that exceeded from their net availability of 86.54 lakh ton from all domestic sources. This gap bridged by import of mainly soybean and sunflower oil, takes them away from the reach of majority population due to their high costs. Inspite of this lacuna, export of oilmeals, oilseeds, and minor oils from India has increased from 5.06 million ton in the financial year 2005–2006 to 7.3 million ton in 2006–2007 in order to hold the agricultural economy. In year 2009, of the 0.6 million ton of sesame seed production, 0.194 million ton was exported (http://fcamin.nic.in/dfpd/EventDetails.asp ?EventId=561&Section=Edible + Oil&ParentID =0&Parent=1&che ck=0). High prices of both soybean and sunflower oil are still a major concern. In light of these aspects from both Indian and global perspective, sesame oil needs to find a strong foothold to meet the demands of a healthy diet. In this constrained scenario, increasing the production of sesame offers some kind of hope, which can be achieved only by overcoming various hurdles limiting the crop yield.

48.3
Constraints on Sesame Productivity

The plant architecture of sesame is poorly adapted to modern farming systems because of its indeterminate growth habit causing nonuniform ripening of capsules, sensitivity to wilting under intensive management, and absence of nonshattering cultivars suited for mechanical harvest [16]. Other physiological and biotic aspects include low yielding varieties and yield losses due to pests. These along with abiotic stresses are responsible for the reduced output, making it a small holder's crop. More importantly, sesame seedlings apparently show slow growth to develop root mass for soil penetration, the duration in which it is susceptible to various pest infestations and fluctuations of soil environments.

48.4
Abiotic Stress and Sesame

Incorporating abiotic stress tolerance in sesame necessitates first and foremost the identification of the important stresses that delimit its productivity. Baseline studies regarding effects of various abiotic stresses have been carried out on sesame, mostly concerned with breeding aspects. Of all the abiotic stresses such as drought, salinity, extremes of temperature, and so on, waterlogging and chilling sensitivity are the two specific abiotic stresses encountered by the cultivated sesame. It does not in any way undermine the need to address other stresses. Although, it is important to note that sesame crop is specifically prone to suffer significant losses if faced with water logging and its cultivation is restricted to areas and duration that are not subjected to low temperatures. However, due to its locally adapted drought tolerance the crop is valuable in many semiarid regions.

48.4.1
Waterlogging Stress

Sesame crop is highly susceptible to waterlogging, as the crop undergoes immediate senescence and declines within 2–3 days of exposure to flooding stress. This causes excessive devastation in fields (Figure 48.3) where accumulated water needs to be regularly drained out. Increased irrigation has shown to significantly reduce the sesame yield [17]. Even drought-tolerant sesame accessions are very susceptible to high moisture [18]. Hence, waterlogging is an important abiotic stress on this crop and there is a need to develop improved genotypes that could survive the flooding stress.

Studies on any aspect of water logging stress on sesame are entirely lacking. However, other crops showing waterlogging susceptibility such as cotton display significant reductions in stem elongation, shoot mass, root mass, and leaf number along with altered expression of 1012 genes (4% of genes assayed) in root tissue 4 h

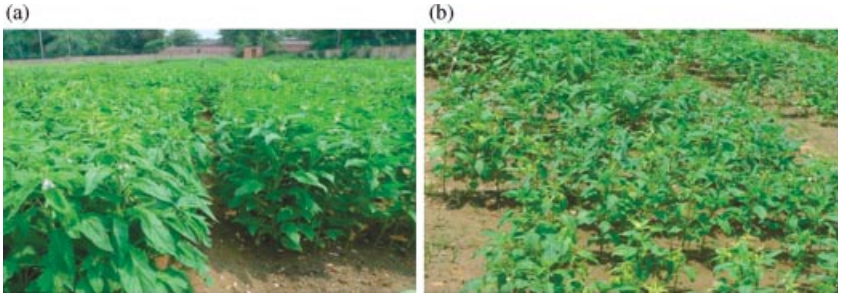

Figure 48.3 Field photograph depicting extreme susceptibility of sesame to waterlogging during *kharif* crop. (a) Well-drained plot (after regular pumping out of stagnant water) showing normal plant growth. (b) Plots left with stagnant rainwater for more than 12 h showing growth retardation, senescence, and plant mortality.

after flooding [19]. Many of these genes were associated with cell wall modification and growth pathways, glycolysis, fermentation, mitochondrial electron transport and nitrogen metabolism. Altered global gene expression was also observed in leaf tissues in response to waterlogging changing 1305 gene expression profiles (5% genes assayed) after 24 h of flooding, mainly involving genes for cell wall growth and modification, tetrapyrrole synthesis, hormone response, starch metabolism, and nitrogen metabolism [19]. Interestingly, in case of rice cultivars, wide variation exhibited for the ability to tolerate complete water submergence was found to be associated with just one locus (Sub 1) on chromosome 9. A transcriptional factor of the B-2 subgroup comprising ethylene-responsive element binding proteins (EREBP) located in the Sub 1 locus could explain the physiological and developmental processes associated with submergence tolerance in rice [20].

Therefore, recent approaches used to develop flooding-tolerant plants are concentrating first on the fermentation pathway. The second focus is specifically on modifying the transcriptional factor gene, AtMYB2, to finally enhance the expression of fermentation pathway genes [21]. However, none of the developed transgenic lines in rice or cotton using this approach has yielded significant tolerance to hypoxic stress. The identification of novel genes from cDNA library of anaerobically induced *Arabidopsis* root [22] that also would be helpful in developing flooding-tolerant sesame genotypes.

48.4.2
Chilling Stress

Sesame comes under the category of chilling (0–15 °C) sensitive plants that include crops such as rice, maize, soybean, cotton, and tomato, where the plants are incapable of cold acclimation, that is, are unable to increase their freezing tolerance when exposed to low temperatures [23]. There is a spontaneous retardation in sesame plant growth observed as drying off of axillary buds and restricted growth of plant organs, namely, leaves, floral buds, and fruits, finally leading to plant death.

Oplinger *et al.* [24] have reported that the absence of 90–120 frost-free days are required for commercial cultivars of sesame where day temperature between 25 and 26.7 °C is favorable. The plant shows significant reduction in growth below 20 °C and growth and germination are totally inhibited below 10 °C. Sesame seed shows marked reduction in content of lignans (sesamin and sesamolin) in the oil [25] during frost damage. This reduced strength of the reactive oxygen species (ROS) scavenging machinery that prevents the system from oxidative stress, generated due to chilling reveals the seriousness of the situation and could be one of the factors responsible for the cellular injury and senescence of the sesame plant.

The mechanism of chilling tolerance, although less worked out, is finding way by the pathways deciphered for cold acclimation as the molecular changes that occur during latter are also found to play a role in chilling tolerance [26, 27]. The few characterized genes basically include the transcription factors that act upstream in cold acclimation or as effector molecules that act to counter the potential damaging effects of cold stress [28]. These cold-responsive genes are members of different low-temperature regulons, as some are regulated by the C-repeat binding (CBF) transcription factor while the others are not [29].

Exogenous application of chemicals such as glycine-betaine has proved fruitful in improvement of tolerance in chilling-sensitive tomato plants [30]. In another approach, chilling-tolerant plants were obtained by increasing levels of trienoic fatty acids [31] or the accumulation of cold-responsive proteins (*COR*) by increased expression of the genes positively regulating their expression such as SCOF-1 from soybean [32], CBF-1 [33], and ABI3 [34]. CBF3 is known to integrate various components of the cold response and its overexpression, in addition to increasing the tolerance of cold-induced accumulation of proline and soluble sugars (reviewed by Yuanyuan *et al.* [35]) [36]. Activation of stress-inducible genes by binding of factors such as DREB1A on the upstream regulatory elements (DRE, dehydration-responsive element) has also led to substantial increase in stress tolerance, including cold stress [37]. Other important category of genes involved in providing cold stress tolerance includes *KIN* (cold-induced), *LTI* (low-temperature-induced), and *RD* (responsive to dehydration) genes. The proteins with multiple hydrophilic ice binding domains termed antifreezing proteins (AFPs), having the ability to inhibit the growth and recrystallization of ice in intercellular spaces [38], are being worked out. The heterologous overexpression of genes encoding AFPs in freezing-sensitive plants enhanced the freezing tolerance of host plants as observed in case of transgenic tobacco plants (made of AFPs of carrot) that survived subfreezing temperature of −2 °C [39].

The role of cellular metabolic signals and RNA splicing, their export, and secondary structure unwinding has emerged out to be of central importance in regulating cold-responsive gene expression and chilling tolerance. One of the key players is ubiquitination-mediated proteasomal protein degradation that has a crucial role in regulating one of the upstream transcription factors, INDUCER OF CBF EXPRESSION 1 (ICE1), and thus in controlling the cold-responsive transcriptome [40]. The knowledge of such key players in plants under cold stress is paving the way for more efficient tools to make them chilling tolerant.

48.4.3 Salt Stress

High salt concentrations limit sesame cultivation especially in arid and semiarid regions. Sesame cultivars show a considerable variation in the degree of salt tolerance [41]. In response to high NaCl treatments (50 and 100 mM), two sesame cultivars Orhangazi and Cumhuriyet showed reduction in root and shoot length, increased lipid peroxidation while dry weights were affected minimally [42]. These affects were more pronounced in the cv. Orhangazi than in cv. Cumhuriyet. Similar studies have shown that dry weight was less affected in salt-tolerant sugar beet and moderately tolerant cotton [43]. Change in free proline levels in relation to salinity have figured out its roles such as balancing capacity as an osmolyte, stabilizing proteins, regulating cytosolic pH, and scavenging hydroxyl radicals [44]. Cultivar Cumhuriyet was superior to the other one in proline content, which increased with time and concentration of NaCl treatment as observed in relatively salt-tolerant plants such as *Beta vulgaris* [45], *Brassica juncea* [46], and alfalfa [47]. Increase in the activities of ROS scavenging enzymes has been found closely related to salt tolerance in case of many plants [48–52]. Constitutive and induced levels of superoxide dismutase (SOD) activity was observed for cv. Cumhuriyet compared to cv. Orhangazi that was accompanied by an increase in the activity of major H_2O_2 scavenging enzymes such as ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POX). This activity declined after 2 weeks that was suggested to be taken care of by lignans (nonenzymatic antioxidative processes in sesame) that also showed increased level of accumulation under stress.

Germinated seedlings of different sesame cultivars grown in the presence of varying concentrations of NaCl (30, 50, and 70 mM) showed alterations in electrophoretic patterns of proteins and other metabolites. Analyses revealed cv. RT-46, RT-54, and RT-127 to be salt tolerant, while cv. RT-125 to be sensitive as it showed retarded seedling growth along with low levels of total soluble sugars, sucrose, SOD activity, and higher malondialdehyde (MDA) and proline content in the presence of more than 30 mM NaCl [53].

During seed germination in sesame, expression of *SeMIPS* (*myo*-inositol 1-phosphate synthase) showed downregulation with increase in concentration and duration of exposure to the saline environment. The protein catalyzes glucose-6-phosphate to *myo*-inositol 1-phosphate, which is the first product in the biosynthetic pathways of myoinositol, phytic acid, and other essential cellular components [54–56]. The *SeMIPS* protein was highly homologous with those from other plant species (88–94%). It was present in several copies and expressed in an organ-specific manner. In case of *Arabidopsis*, Nelson *et al.* [57, 58] have also shown salinity to affect transcription of the *MIPS* gene during biosynthesis of *myo*-inositol and its derivatives. Similar salinity stress studies on *Arabidopsis thaliana* showed its upregulation in salt-tolerant plants and its reduction in the salt-sensitive ones [59]. Downregulation of EST coding for *MIPS* was also observed in sunflower (a salt-sensitive crop), under salt stress [60]. Genes responding to salinity have been reported from rice [61], common ice plant (*Mesembryanthemum crystallinum*) [62–64], and tomato [65], which could be implemented in achieving salt tolerance.

48.4.4 Drought Stress

Sesame is known to be endowed with the property of drought tolerance due to its extensive root system. However, drought severely limits sesame production in marginal and low rain-fed areas. In addition, accessions from wet areas such as Korea and Bangladesh are very susceptible to drought [18]. Assessment of drought tolerance as a ratio of yield under water stress to that under normal irrigation carried out for 17 sesame genotypes showed that the seed yield was sensitive to water shortage more than the morphological characters [66], while the mean weight of individual seeds escaped such effects. This depicts that postflowering response of drought in sesame is production of less seeds, instead of compromising on the seed size [67]. A survey of 27 sesame genotypes in Moghan region for tolerance to drought revealed Karaj1, Naz takshakhen, Varamin237, and Varamin2822 genotypes to be mid-resistant and suitable for cropping under drought stress on the basis of six drought tolerance indices [68].

Assessment of enzymes involved in prevention of ROS generated as an outcome of drought stress revealed increased activities for SOD, POX, CAT, and polyphenoloxidase (PPO) both in leaves and roots of sesame [69]. On the other hand, fresh and dry mass and total protein content of leaves showed a decreasing trend. Assay for MDA content was indicative of reduced lipid peroxidation. Yekta cultivar of sesame was found to be more resistant than the cultivar Darab14 [69].

Improved tolerance against drought is reported to have an association with increased content of cuticular waxes per unit leaf surface area in oats, rice, sorghum, alfalfa, and crested wheat grass [70]. However, the increased wax deposition does not always correlate inversely with transpiration rate. Imposition of water deficit on sesame cultivars caused an increase in wax amount by 30%, with 34% increase in alkanes, 13% in aldehydes, and 28% of the unknown ones [67]. An increase of 49% in cuticle thickness due to monomers of alkanes has also been observed in *Arabidopsis* plants on subjection to water deficit. Under such conditions, the gene ECERIFERUM1 having role in alkane metabolism showed upregulation [70]. Transcriptional factors such as WAX INDUCER 1 (WIN 1) in *Arabidopsis* have also been implicated in increasing wax deposition [71]. Deciphering of drought induction of alkane metabolic pathway, role of cuticle, and the actual mechanism behind slowing down of transpiration rate in drought tolerance, which regulates survival of sesame plant, is still awaited.

48.4.5 Heavy Metal Stress

Previous investigations have found oil-yielding plants, namely, mustard and sunflower, to be suitable for rhizoextraction as they accumulated more Cr from the soil in comparison to 36 other agricultural plant species [72]. Within oilseeds the pattern of heavy metal accumulation showed sesame to stand third after peanuts and rapeseed with sunflower being the last. The distribution pattern in the plant organs of sesame

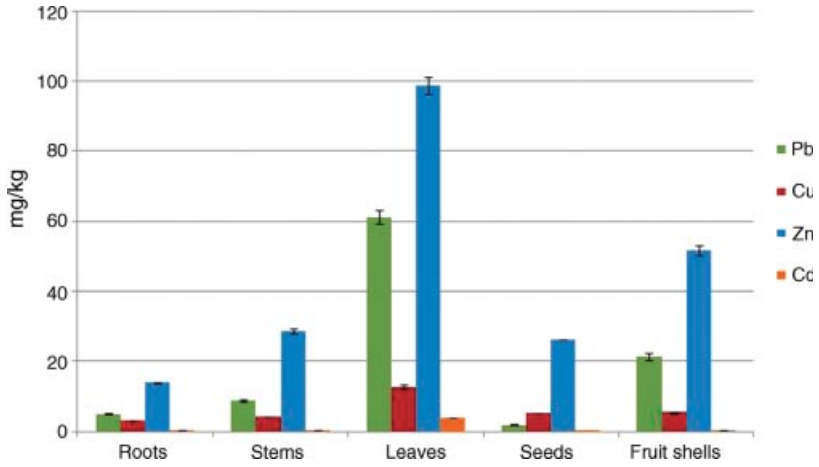

Figure 48.4 Organ-wise distribution of heavy metals in sesame at commercial ripeness stage (adapted from Angelova *et al.* [73]). Error bars represent the standard deviation.

showed an order of leaves > stem > roots > fruit shell > seeds [73] (Figure 48.4), revealing that the content was very less in the economically important edible organ, the seeds. The respective accumulation of heavy metals after 60 days of growth period in sesame followed an order of K > Na > Fe > Zn > Cr > Mn > Cu > Pb > Ni > Cd as determined by the technique of sequential extraction using EDTA [7]. In addition, the translocation of these metals was found less in upper parts. The potential of sesame to extract heavy metals from contaminated soils has also been determined by chemical fractionation analysis of *S. indicum* var. T55, which depicted an increased accumulation of toxic metals (Cr, Ni, and Cd) with increase in sludge ratio. The tannery sludge favored plant growth at lower amendment rates (25%) as depicted by increased fresh weight and number of leaves [74].

Increased affinity of sesame seeds toward accumulation of trace elements has also been reported in comparison to corn grains (0.54–1.94 ppm). Range of Cr content in sesame seeds was 0.77–2.14 ppm, with recommended daily allowances being 0.05–9.2 ppm [75]. Application of organic waste compost including municipal waste (MW) had no effect on the chemical constituents (oil, carbohydrates, and total protein) of sesame seeds. With treatment of MW, lead and cadmium concentrations increased in the plant; however, the amount of metals tested did not exceed the phytotoxic level [76]. Seeds of the high-yield sesame cultivar PB-1 showed significant tolerance to lead as shown by accumulation of more dry mass during early growth phase, although fresh weight showed slight inhibition at higher levels (2 mM of Pb^{2+}). On the other hand, cultivar HT-1 was shown to be Pb^{2+} sensitive [77]. Accumulation of Pb^{2+} increased with increasing concentration of the heavy metal in root, stem, and leaves. Estimation of *in vivo* nitrate reductase activity showed inhibition in roots and leaves with less effect on the latter, correlating with the respective accumulation of Pb^{2+} in the organs. However, *in vitro* nitrate reductase activity was not inhibited by

the metal. Total organic nitrogen was higher in young roots proposed due to increased N translocation from seeds to roots [77]. All these studies suggest that sesame shows tolerance to the presence of heavy metals in the soil and in fact shows significant ability to accumulate such elements. Therefore, sesame plant provides an interesting research system to understand the mechanisms involved in heavy metal accumulation, an area that is gaining importance for dealing with the management of degraded ecosystems due to contamination by undesirable concentration of toxic metals. However, it is of utmost importance to ensure the complete absence of these harmful moieties in the seed and seed oil for the safe edible consumption.

48.5
Abiotic Stress and Areas of Special Focus in Sesame

Out of the vast repertoire of genes governing abiotic stress tolerance in plants, few genes and gene products have gained special focus in sesame.

48.5.1
Lipid Transfer Proteins

The transfer of phospholipids between membranes is facilitated by group of proteins called lipid transfer proteins (LTPs) [78]. These are small (7–10 kDa), abundant, basic proteins containing eight conserved cysteine residues involved in four disulfide bridges. On the basis of the number of amino acids between the fourth and fifth cysteine residues in the motif's core, plant LTPs have been categorized into eight groups (LTP1–LTP8) [79]. Initially observed in spinach leaves, maize coleoptiles, and barley aleurone layers, they show ubiquitous expression in seedlings, leaves, stamen, tapetum, and microspores, as well as in somatic and zygotic embryos [78]. LTP genes are shown to be responsive to environmental changes such as salt, drought, abscisic acid (ABA) or cold treatment [80–84]. A nonspecific LTP gene isolated by subtractive hybridization between drought-tolerant and -sensitive sunflowers showed transcript induction by water stress and ABA [85]. cDNA microarray analysis in sunflower revealed upregulation of an LTP under chilling stress. However, downregulation of LTP was exhibited in saline environment as earlier shown in case of *Arabidopsis* [86]. LTPs are proposed to have a function in repairing stress-induced damage in membranes or changes in their lipid composition, perhaps to regulate their permeability to toxic ions and the fluidity [87, 88]. LTPs have also been shown to be secreted in response to NaCl stress and even affect cell wall extensibility [89]. Periodic dehydration stress in tree tobacco (*Nicotiana glauca*) leaves increased LTP mRNA expression and cuticular wax deposition. In addition, immunolocalization and LTP:: GFP fusion studies have localized LTPs to the cell wall and predominant expression in the epidermis [90, 91] suggesting their role in cutin and wax assembly [92]. Most plant LTPs are secreted to the cell wall by unidentified pathways. Although some intracellular LTPs have been observed in glyoxysome matrix of castor (*Ricinus comunis*) seed and protein storage vacuoles of cowpea (*Vigna unguiculata*) [93, 94].

These LTPs are present in multiple isoforms as detected in *Arabidopsis* and rice genome, showing differential expression pattern, whose functions are yet to be discovered [79, 83].

It is important and pertinent to point out that LTPs constitute one of the most abundant ESTs of immature sesame seeds of which 21 isoforms were identified [95]. Partially purified LTP isoforms of sesame (*SiLTP*) showed the ability to bind fluorescent fatty acids and transfer fluorescent phospholipids. Five *SiLTP* isoforms were most abundantly expressed in developing seeds, but also detected in flower tissues. *SiLTP3* and *SiLTP4* transcripts were also expressed in leaves and seed walls, respectively. *SiLTP2* and *SiLTP4* isoforms exhibited significantly inducible expression patterns with exogenous application of 300 mM NaCl and 300 mM mannitol. Exogenous ABA, which has been shown to mediate plant tolerance to water or cold stresses, also significantly induced the *SiLTP2* and *SiLTP4* isoforms. These were localized to plant cell membranes as seen by transient expression in *Arabidopsis* and were found to be associated with large organelles such as ER, probably being secreted from the cells via the classical secretory pathway. However, the biological roles of *SiLTPs* in response to salt and osmotic stresses remain to be elucidated, which might play an important role in plant acclimation to water stress during seed development.

48.5.2 Caleosins

Caleosins are calcium binding proteins of 27 kDa, ubiquitous among higher plants with similar candidates in algae and fungi [96]. These were identified in sesame seeds as minor proteins (Sop1) of oil bodies (OB) by immunolabeling [97]. Sop1 was found to be homologous to a rice protein (*OsClo*) that expresses abundantly in late embryogenesis and is responsive to ABA and osmotic stress (dehydration and NaCl) in seedlings and in vegetative tissues [98]. Similarly, mRNA of an *Arabidopsis* caleosin homologue (*AtClo1*) was detected in response to ABA during dehydration [99]. Sesame caleosin (*SiClo1*) mRNA accumulates in OBs, with maximal expression seen 2 weeks after flowering, thereafter undetectable in mature seeds [100]. In contrast to *OsClo*, *SiClo1* is therefore apparently seed specific. Recent analyses indicate that *Arabidopsis* contains at least five caleosin-like genes situated on four of the five chromosomes (*AtClo1–5*, [96]). *AtClo2–4* are expressed at low levels in various tissues, including nonoil storage tissues, while *At-Clo1* expression is seed specific. Interestingly, *AtClo1–4* expression is not responsive to ABA or osmotic stress in vegetative tissues, as in case of rice *OsClo*. Carrot oleosin is responsive only to ABA in embryonic tissue, and not in adult tissue [101]. A better understanding of this expression pattern in plant stress responses would be beneficial.

Caleosins have been located on the surface of OBs or associated with an endoplasmic reticulum (ER) subdomain [96]. Caleosin comprises three distinct structural domains: a unique N-terminal hydrophilic domain (containing a single Ca^{2+} binding EF hand), a central hydrophobic anchoring domain, and a C-terminal hydrophilic domain containing four possible phosphorylation sites [98, 100]. The central hydrophobic domain of *SiClo1* is proposed to consist of an amphipathic

α-helix followed by a short anchoring region formed by a pair of antiparallel β-strands connected by a proline knot-like motif [100]. The EF hand of *OsClo* is known to bind calcium *in vitro* [98]. Similar probability exists for *SiClo1* to bind calcium as *SiCLO1* purified from oil bodies or heterologously expressed exhibits EGTA-retarded and calcium-rescued migration in SDS-PAGE [100].

An *Arabidopsis* caleosin, *RD20* (responsive to dehydration 20) is reported to show enhanced expression by ABA, salt, dehydration, and osmotic stresses [99, 102]. It is among the early induced genes, one of the most highly expressed and often used as a stress marker gene [103, 104]. *RD20* is located at the bottom of chromosome 2, a region that appears to be important in the regulation of plant transpiration efficiency in *Arabidopsis*. It is expressed in particular tissues or organs during plant development. In contrast to other *Arabidopsis* caleosin genes *RD20* promoter sequence is enriched with AtMYC2 binding sites such as the ABRE (ABA-responsive element), ABRE-like, and DRE. Interestingly, AtMYC2 acts as a positive regulator of ABA signaling under drought stress [105]. *RD20* is able to bind calcium and also support a putative peroxygenase activity as in case of *OsClo* and *AtClo1* [99, 106]. Such peroxygenase activity in maize is involved in cuticle and wax synthesis [107], the latter is enhanced under water deficit conditions and ABA, to prevent water losses [70]. Recently, *RD20* was shown to be one of the components involved in enhancing tolerance to water deficit mechanisms through the regulation of stomatal aperture, plant growth, and water use efficiency [108] and in salt stress response, thus, hypothesized to act as a stress signaling hub that sets up multiple abiotic responses. The ability of sesame caleosins to undergo ubiquitination at two sites in the lysine residues in the C-terminal domain has been reported [109]. As ubiquitination of proteins has also been observed in stress responses [110], it would be interesting to establish such a role, if any, for *SiClo*.

48.5.3 Steroid Dehydrogenase

Sesame steroleosin (Sop 2), a 39 kDa protein comprising 348 amino acid residues, is a minor protein of OBs [111]. The Sop2 gene was obtained by immunoscreening that showed transcription in the maturing seeds. The protein possesses a hydrophobic anchoring segment preceding a soluble domain homologous to sterol binding dehydrogenases/reductases known to be involved in signal transduction in diverse organisms. Structure of the soluble domain consists of a seven-stranded parallel β-sheet with the active site, S-(12X)-Y-(3X)-K, between an NADPH and a sterol binding subdomain. Its sterol-coupling dehydrogenase activity has been demonstrated both in the overexpressed soluble domain of steroleosin and in purified oil bodies. Southern hybridization suggests the presence of one steroleosin gene and certain homologous genes in the sesame genome [111]. In contrast, eight hypothetical steroleosin-like proteins are present in the *Arabidopsis* genome with a conserved NADPH binding subdomain, but a divergent sterol binding subdomain. Steroid dehydrogenase-like protein (SDs) has been found to be induced by drought stress [112] and in response to high light stress and ascorbate deficiency in

Arabidopsis [113]. This indicates role of SDs in protecting against ROS produced by high light and in the absence of ascorbate (vitamin C), one of the major antioxidant species of chloroplasts that is a cofactor of thylakoid-bound and stromal ascorbate peroxidases that detoxify H_2O_2 produced by SODs. Transgenic *Arabidopsis* plants overexpressing hydroxylsteroid dehydrogenases (AtHSD1) also provide increased tolerance to salinity stress [114]. Whether this tolerance is mediated by brassinosteroid signaling or increased ABA catabolism remains to be elucidated, before getting utilized in other crops such as sesame.

48.5.4 Phytostatins

Phytocystatins, homologues of cystatins of animals, are a small family of plant proteins commonly ranging from 12 to 16 kDa, consisting of more than 80 members [115]. In general, they possess three conserved residues, a G residue in the vicinity of the N-terminal end, a highly conserved QVVAG motif in a central loop segment, and a PW dipeptide motif closer to the C-terminal end of the protein, which are known to interact with the active site cleft of cysteine proteinases belonging to the papain family, causing their reversible inhibition [116]. In addition, plant cystatins possess a unique and conserved LARFAVDEHN sequence at the N-terminal end of an α-helix segment [117]. Apart from their participation in biotic stresses and seed germination, their role in abiotic stresses has been proposed. Accumulation of phytocystatin mRNA (AtCYS1) has been observed in *Arabidopsis* under high-temperature stress [118], in the vegetative tissues of barley plants subjected to anaerobiosis, darkness, and cold shock [119], and in the leaves and roots of chestnut plantlets exposed to cold, saline- or heat-stress [120]. Similarly, in grain amaranth, increased expression of cystatins (AhCPI) was seen in roots and stems substantially in response to water deficit, salinity, cold, and heat stress, whereas heat stress induced a rapid and transient accumulation in leaves [117].

Low-abundant endogenous cystatin (22 kDa) has been purified to homogeneity via a papain-coupling affinity column from mature sesame seeds. These were shown to express in germinating seeds; however, their ability to inhibit endogenous cysteine proteases was not revealed [121]. These proteins from sesame have been expressed and purified via artificial oil bodies for final biotechnological application in protection of plants and other industrial uses [122]. The role of sesame phytocystatins in abiotic stresses requires further research efforts.

48.5.5 Lignans

Stresses such as drought, salt, UV radiation, ozone, chilling, heat shock, pathogen attack, and so on increase the production of ROS in plants leading to development of several enzymatic and nonenzymatic defense systems against ROS. Under such oxidative stress conditions, plants with high constitutive and induced antioxidant levels are known to have better resistance to damage [123–125].

In contrast to the meager knowledge on enzymatic antioxidative defense system operating in sesame, the nonenzymatic antioxidant substances termed lignans effective in oxidative defense system are well studied. Widely distributed in vascular plants, lignans comprise a large group of natural products characterized by coupling of two *p*-hydroxyphenylpropane (C_6C_3) units and possessing a β,β′-linkage. These are represented by about 20 compounds in sesame, basically in the form of oil-soluble and glycosylated lignans [126]. Among the oil-soluble ones, the most predominant are sesamin and sesamolin, accompanied by traces of others. Interestingly, the two lignans, sesamolin and sesamolinol, possess an oxygen bridge between their benzene and furofuran rings, the feature unique to the genus *Sesamum* [127]. Sesame seeds have on average 0.63% lignans with sesamin and sesamolin contents ranging from 0.07 to 0.61% (mean ± SD, 163 ± 141 mg/100 g) and from 0.02 to 0.48% (101 ± 58 mg/100 g), respectively [7, 128]. Among sesame cultivars of India, those collected from the Northeastern states are found to be higher in lignan content (18 g sesamin/kg, 10 g sesamolin/kg) [129]. The most frequent technique used for lignan-type detection and quantification in sesame is high-performance liquid chromatography [130–132] that has now been subsequently simplified [133]. LC-NMR-MS has also emerged as the fast screening device for the characterization of sesame lignans from various sesame oil-based sources, for assessing the antioxidant activity of the extracts, and especially for modifying the lignan profile of the conventional sesame oil extract to get a sesaminol-enriched extract [134].

Studies on these lignans with respect to stress have displayed their stress combating ability. Under *in vitro* experimentation, sesaminol was shown to inhibit Cu^{2+}-induced lipid peroxidation in low-density lipoprotein and was found to be more effective scavenger than either α-tocopherol or probucol in reducing the peroxyl radicals in aqueous solution [135]. The analysis of sesame oil extracts for antioxidant activity has revealed the following order: sesamin < sesame oil extract < sesaminol-enriched sesame oil extract < sesamol. The combination of sesaminol and γ-tocopherol has been proposed to be synergistically responsible for the actual oxidative stability of sesame oil [134].

Sesamin, one of the major furofuran lignan in sesame seeds, has been extensively studied by Ono *et al*. [136]. The gene responsible for the catalysis of sesamin biosynthesis from pinoresinol (the first lignan in the pathway) is deciphered out to be a cytochrome SiP450, CYP8101. The protein requires NADPH for its activity and is unique due to its dual catalyzing ability leading to the formation of two methylenedioxy bridges (producing sesamin via piperitol), which is restricted to only one such bridge in all known P450 proteins. This biosynthesis has been localized to the cytoplasmic surface of endoplasmic reticulum as revealed by the expression of CYP81Q1-GFP protein in onion epidermal peel via transient system. The gene is present singly and its functional validation has been obtained by the observation of a functional homologue isolated from *S. radiatum* (having sesamin in seeds) and the presence of a nonfunctional P450 homologue from *S. alatum* that lacks sesamin. The P450 proteins are shown to have evolved independently as the CYP81Q1 shows only 24% sequence identity with the Ranunculaceae member, *Coptis* CYP719A1. The mode of action of CYP81Q proteins has also been proposed, the diagrammatic

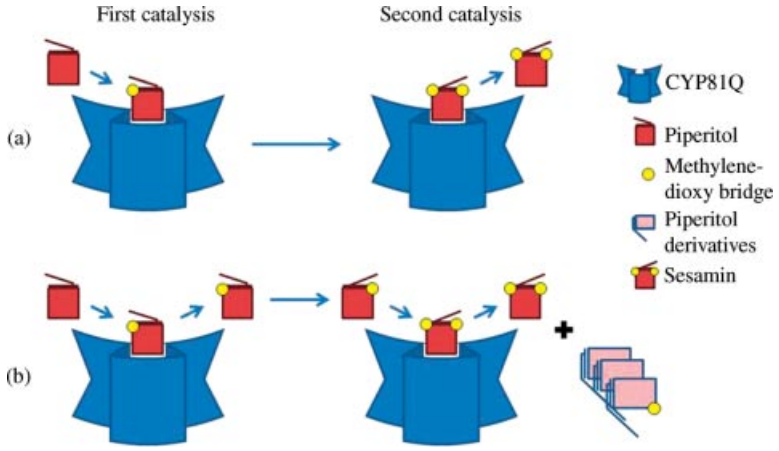

Figure 48.5 Diagrammatic representation of the two alternative models proposed for the mode of action of cytochrome P450 of sesame, CYP81Q (adapted from Ono *et al.* [136]). (a) Sequential methylenedioxy bridge formation. Piperitol reverses in the active site after the first catalysis for formation of second methylenedioxy bridge at the opposite end. (b) Piperitol is released after first catalysis and then recaptured for second one, a part of it being converted to its derivatives.

representation of which is given in Figure 48.5. Genes involved in biosynthesis of antioxidant lignans and accumulation of storage products have been identified in the form of 3328 ESTs from a cDNA library of immature seeds [137]. When compared to the model plant *Arabidopsis* proteome, a total of 62 ESTs from sesame were proposed to be involved in lignan biosynthesis (Figure 48.6).

Apart from being beneficial to human health as anticancerous and anticholestrol agents, studies on rats showed lignans to increase the expression of β-oxidation-associated enzymes in peroxisomes (upregulation of 38 genes, 16 of which are involved in lipid metabolizing function) [138]. Several mechanisms of action have been proposed to explain the potential physiological role of sesame lignans. Especially in animal systems, namely, cultured liver cells. The role of sesamin on the lipid metabolism has been studied extensively and the following mechanisms of actions have been proposed: inhibition of Δ5-desaturase activity [139], inhibition of HMG-CoA reductase activity [140], and inhibition of acyl-CoA cholesterol acyltransferase (ACAT) activity [141]. However, such gene profiling studies and their potential status associating lignans with different stresses are lacking with respect to plants as a system. Overall, this area needs further attention to decipher the actual mechanism behind the combating of stress in plants in general and sesame in particular.

48.5.6
Geranylgeranyl Reductase

Tocopherols are lipid-soluble antioxidants known collectively as vitamin E. These compounds are the major line of defense against ROS generated during various

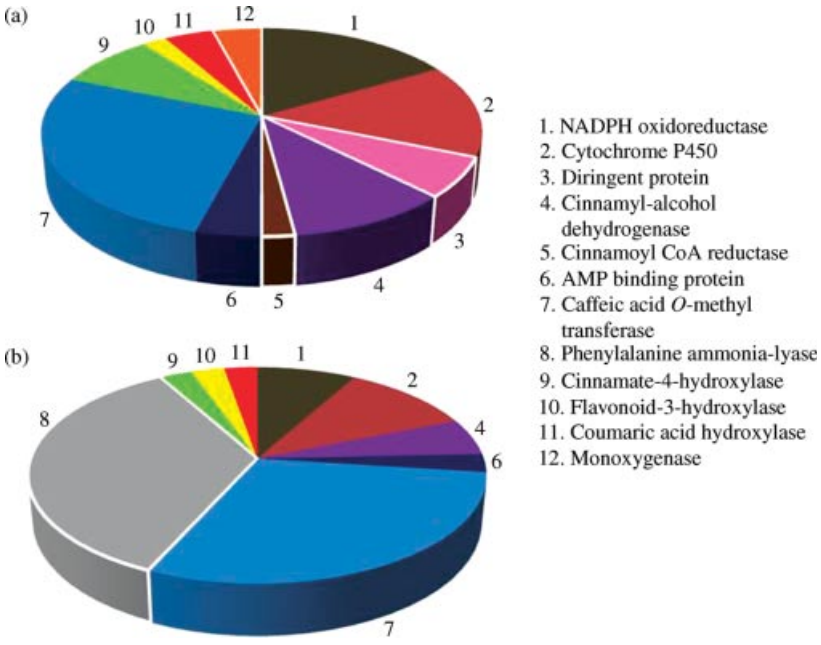

Figure 48.6 Putative ESTs involved in synthesis of lignan, an innate antioxidant defense system in plants (a) sesame and (b) *Arabidopsis* (adapted from Suh *et al.* [137]). Sectors unique to the respective plant are highlighted with white borders.

abiotic stresses. Plants synthesize four isoforms of tocopherols, α-, β-, γ-, and δ-tocopherol, which differ by the numbers and positions of methyl substituents on the aromatic rings of the molecules [142]. The highly efficient antioxidant sesame oil contains about 528 μg/g of total tocopherols [143]. Formation of the first tocopherol intermediate requires reduction of geranylgeranyl diphosphate (GGPP) to phytyl diphosphate [144] that is catalyzed by the enzyme GGPP reductase (*Chl P*) [145]. Sesame GGPP reductase (*SiChl P*) encoded as a 465 amino acid polypeptide shows high degree of similarity to the plant *Chl P*s with the closest evolutionary relationship to tobacco *Chl P* [146]. In contrast to the bacterial *Chl P*s, the *SiChl P* contains an aminoterminal extension that resembles a plastid transit peptide sequence, indicating the possible localization of the enzyme in chloroplasts as known for plant *Chl P*s [147]. The sequence consists of 55 amino acids with the cleavage site located between A55 and A56 in the sequence NLR → VAV. This transit peptide predicted from *SiChl P* is rich in serine and threonine but deficient in acidic amino acids [148]. GGPP belongs to the family of oxidoreductases that contain a nucleotide cofactor-binding domain for stabilization of the β-strand and α-helix interaction, connected by a short loop in which the ligand binding domain is located [149]. The presence of typical motif commonly found in oxidoreductases, V/IXGX1- 2GXXGXXXG/A, in the N-terminus of the mature *SiChl P* polypeptide clearly indicates its function [146]. *SiChl P* is present as a single gene with its high expressions observed in developing

seeds and leaves supporting its role in tocopherol biosynthesis in sesame. In addition to factors such as dark and ethylene, *SiChl P* was shown to be repressed by ABA, which is generally produced upon drought and cold stress with subsequent induction of expressions of various subsets of downstream genes [150, 151]. Similar diminished expression was seen for *PpChl P* in peach leaves in response to cold stress and wounding [152]. *Chl P* has been identified from soybean, ice plant, and several photosynthetic bacteria, although detailed investigations have been performed only in a few plant species such as tobacco [145] and *Arabidopsis* [153].

48.5.7 γ-Aminobutyric Acid

γ-Aminobutryric acid (GABA) is a four carbon nonprotein amino acid found in all prokaryotic and eukaryotic organisms. The metabolic pathway involving synthesis and catabolism of GABA is known as "GABA shunt," as it bypasses two steps of tricarboxylic acid cycle. It rapidly accumulates in responses to heat, drought, salt and low-temperature stresses [154–157]. GABA is proposed to be involved in stress perception in sesame, as application of stresses such as drought, salt, heavy metal, and high temperature showed increment in GABA levels, but it was not able to sustain normal plant growth [158]. Highest increment of GABA was in case of heavy metal treatment, followed by drought, while accumulation rates under salt and high-temperature treatments were almost the same. Differential ability of the sesame plant in coping with these stresses was proposed to be the reason behind variation in the rate of GABA synthesis [158]. Upregulation of a GABA receptor has been observed in two tolerant genotypes of barley [159] and increased expression of genes for GABA shunt in response to drought [160]. GABA has been shown to reduce accumulation of ROS in aluminum and proton-stressed barley [161]. Its other proposed roles under different stresses include maintenance of C:N balance, regulation of cytosolic pH, and osmoregulation and as a signaling molecule [162]. Furthermore, the role of Ca^{2+}/CaM in GABA-mediated tolerance to oxidative stress, heat shock, and osmotic and salt tolerance through effector molecules has also been suggested [163].

48.5.8 Metallothioneins

Metallothioneins (MTs) are ubiquitous low-molecular weight, cysteine-rich cytoplasmic proteins that can bind metals via mercaptide bonds. On the basis of the number and arrangement of cysteine residues, all the plant MTs belong to class II (in contrast to the vertebrate class I) and can be further subdivided into four types according to the distribution pattern of Cys residues [164, 165]. Expression analysis has shown type 1 MTs to express preferentially in roots [166], type 2 mainly in leaves [167, 168], type 3 in ripe fruits and leaves [169], and type 4 in developing seeds [170–172]. In plants, MTs are known to participate in maintaining the homeostasis of essential copper (Cu) and zinc (Zn) at micronutrient levels and in heavy metal stress for detoxification of nonessential toxic metals such as cadmium

(Cd) and arsenic (As) [173–176]. The expression of MT genes is affected by oxidative stress, and other abiotic stresses, such as drought and salt [177, 178], hence their role as protectants from oxidative damages has been proposed [179–182]. MTs from *Tamarix hispida* (*ThMT3*) was upregulated by high salinity and heavy metal ions, with predominant expression in the leaf [183]. Transgenics for MTs in rice (*OsMT1a*) enhanced its drought tolerance and increased activity of ROS scavenging enzymes [184] showing expression in roots. Tobacco plants overexpressing *GhMT3a*, a type 3 MT isolated from cotton (*Gossypium hirsutum*), were shown to have reported only half of H_2O_2 levels in transgenic than those in wild-type plants under salt, drought, and low-temperature stresses, suggesting that changes in ROS signaling might be the reason for higher stress tolerance [185]. It has been hypothesized that MTs scavenge the superoxide radicals either independent of SOD or act as an activator of SOD by supplying metals such as Cu or Zn to apo-SOD [186]. Transcriptome analysis of mature sesame seeds has revealed the presence of abundant transcripts for metallothioneins [171] indicating the presence of a conserved metal toxicity combating mechanism in sesame. Further research on overcoming the limitations regarding *in vivo* protein expression studies would be beneficial in dissection of the actual process of scavenging performed by the MTs.

48.6
Approaches for Incorporation of Abiotic Tolerance

The process of incorporation of genes imparting tolerance to abiotic stresses would involve either avoiding the stress or combating the stress by inducing expression of genes that directly or indirectly are responsible for the synthesis of required metabolites. The basic strategies initiating synthesis of these metabolites can be targeted either at the functional genes or regulatory genes. Advances in high-throughput techniques have enabled identification of large number of genes that are differentially regulated in response to a specific environmental stress. Both conventional and nonconventional methodologies await their utilization for incorporating abiotic stress tolerance in sesame. The final goal of developing stress-tolerant genotypes can be achieved by different approaches, but the key steps that need to be undertaken (Figure 48.7) are as follows:

- Search for useful traits/genotypes.
- Identifying and understanding the precise mechanism and genes responsible for the stress tolerance.
- Transfer of the trait using conventional or biotechnological tools.
- Screening of the target genotypes for successful transfer of the desired trait.
- Acceptance of the farmers for growing the novel genotypes.

Therefore, irrespective of the approach used for incorporation, the first and foremost requirement is the search of the donor of the desired trait. Conventional approaches can be applied only if taxa having the desired trait constitute the primary, secondary, or even tertiary gene pool of the crop plant. In the absence of desired trait

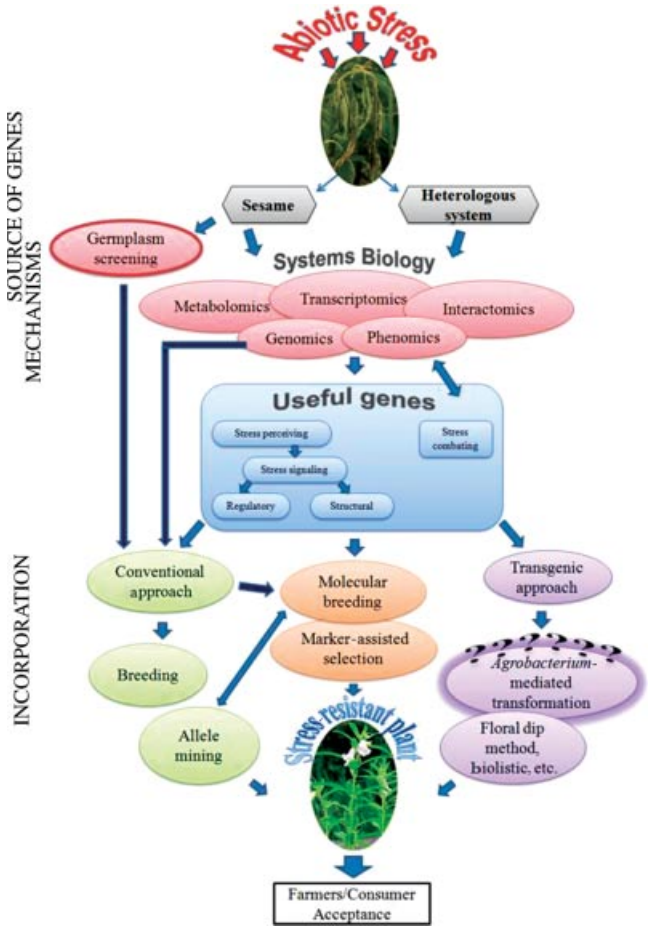

Figure 48.7 Schematic representation of the fundamental steps involved in development of abiotic stress-resistant plants using different approaches.

within the conventional gene pool, the biotechnological approaches provide the required tools for the transfer of the traits from the donor taxa to the desired background. Once the transfer has taken place, the screening methodologies would remain the same in both the approaches. However, the acceptance of the improved cultivar by the farmer and the consumer can again have different reactions depending upon the approach used.

48.6.1 Search for Useful Genes in the Sesame Germplasm

Natural genetic diversity is a sustainable resource that can enrich the genetic basis of cultivated genepool with novel alleles that improve productivity and adaptation.

Substantial genetic variation existing in the cultivated genepool of sesame can be exploited in breeding programs addressing abiotic stress tolerance. Furthermore, the wild species of *Sesamum* have been reported to possess desirable genes for the characters of major importance in sesame breeding including drought resistance and tolerance to heavy rainfall [187]. Desirable characters have been identified for the sesame improvement with aims to maintain and enhance sesame production in addition to high yield potential coupled with harvest index, seed retention, uniform maturation through determinate habit, and tolerance to biotic and abiotic stresses. A list of major characters identified [6, 188–192] with an emphasis on overcoming abiotic stresses are listed below.

Seedling characters: Fast vigorous germination and emergence with strong hypocotylar elongation, rapid growth in early stages, and ability to germinate and withstand lower temperatures.
Plant characters: Rapid root growth, deep taproot penetration with well-distributed secondary root system; leaves with medium to broad base, narrow lanceolate toward apex, short petioles, higher photosynthetic efficiency, and early abscission.
Physiological characters: Photo- and thermoinsensitivity, early maturity, and higher nutrient uptake under low fertility; tolerance to water logging, drought, salinity; nonlodging under high fertility; and uniform ripening.
Yields: High and stable under a wide range of environmental conditions.

Significantly large germplasm collections have been made for sesame by different sesame growing countries and have been characterized for morphoagronomic traits following the IBPGR (now Biodiversity International) descriptors.

Being the center of origin and diversity for sesame [4], India has particularly rich diversity for economically important traits that are largely underexplored for use in crop improvement programs [5, 193]. However, a systematic screening of the germplasm or even the core collections identified is entirely lacking. Few fragmented studies using limited collections have been attempted for tolerance to different abiotic stresses. Therefore, there is need to undertake systematic and comprehensive screening of sesame germplasm for tolerance to individual abiotic stresses giving suitable environment. The specific trait-based core collections can be identified and used as reference collections for individual stresses. The promising germplasm accessions identified can be directly used as the donor in the conventional breeding programs and also subjected to detailed investigations for gene prospecting.

The major challenges in successful incorporation of abiotic stress tolerance in the desired taxa or genotypes are the gaps in the understanding of the mechanisms responsible for their expression. First, the precise metabolic and structural attributes that impart such valuable adaptations in the plants are far from understood. Next major challenge is the transfer of these traits to the genotypes of our choice. A synergy between traditional breeding and genomic approach is the need of the hour in meeting these challenges. Characterization of sesame genetic diversity should employ the tools of functional genomic approaches. Therefore, discovery of gene trait from the diverse genetic resources available coupled with phenotyping and

bioinformatics followed by proof of the candidate gene function *in planta* leading to successful expression of the trait in the desired background is being advocated [194]. Growth or yield penalty in the stress-resistant plants under the unstressed conditions is yet another challenge that can be addressed by driving expression of genes in response to stress by an inducible promoter [195].

48.7 Conventional Approach

The conventional approaches to develop the genotypes of interest involve creation of new gene combinations by either crossing the genotypes having the desired traits individually or by introducing new germplasm. The desired variability thus obtained needs to be narrowed down to few genotypes. The success of the effort necessitates exercising high selection intensity (i.e., selection of few genotypes) on genotypes having large differences (i.e., having high genetic variance) using accurate methods to assess characters that are transmitted to subsequent generations (i.e., having high heritability).

Conventional breeding approaches for incorporation of the desired trait have always been the first choice of the breeders provided the source of the desired gene and the target genotypes do not pose uncircumventable crossability barriers. This approach essentially requires identification of stress-resistant genotype within the gene pool, followed by their crossing with agronomically superior cultivar and repeated backcrosses and selection of the desired phenotypes in each generation. Although attempts are being made to develop drought-tolerant sesame cultivars through breeding approach [66], the process is challenging because of gaps in understanding/information on precise phenotypic traits to be selected in the segregating generation. This process can be made more specific and targeted by marker-assisted breeding. However, there are still no such usable validated markers available in sesame. Furthermore, the very basic requirement of MAS, that is, a saturated linkage map based on molecular marker, is still lacking and is a prerequisite for background selection for recipient's genotypes.

48.7.1 Recent Approaches in Utilization of Genetic Variation

48.7.1.1 Association Genetics

Association mapping, a high-resolution method for mapping quantitative trait loci, based on linkage disequilibrium, holds great promise for the dissection of complex genetic traits. Most traits related to abiotic stress tolerance are controlled by multiple quantitative trait loci. Genetic mapping and molecular characterization of these functional loci would facilitate genome-aided breeding for stress tolerance. Two of the most commonly used tools for dissecting complex traits are linkage analysis and association mapping. Linkage analysis exploits the shared inheritance of functional polymorphisms and adjacent markers within families or pedigrees of known

ancestry. Linkage analysis in plants has been typically conducted with experimental populations that are derived from a biparental cross. Although based on the same fundamental principles of genetic recombination as linkage analysis, association mapping examines this shared inheritance for a collection of individuals often with unobserved ancestry. As the unobserved ancestry can extend thousands of generations, the shared inheritance will persist only for adjacent loci after these many generations of recombination. Essentially, association mapping exploits historical and evolutionary recombination at the population level [196]. By exploring deeper population genealogy rather than family pedigree, association mapping offers several advantages over linkage analysis such as much higher mapping resolution, greater allele number, broader reference population, and less research time in establishing an association. Linkage analysis and association mapping, however, are complementary to each other in terms of providing prior knowledge, cross-validation, and statistical power.

The linkage disequilibrium approach that forms the basis for association mapping is useful in identifying the genetic mechanisms responsible for abiotic stress tolerance and their fine dissection. Linkage disequilibrium (LD) refers to the nonrandom association of alleles between genetic loci. The core collection of sesame representing the genetic variation in cultivated sesames [197] is a good source for identifying the basic mechanisms contributing to abiotic stress tolerance in sesame. The process is also useful in detection of molecular markers closely linked to the genes of interest, thereby providing a mechanism to transfer the genes identified from donors to recipients through marker-assisted breeding programs.

48.7.1.2 **Phenomics**

Precision of association analysis depends to a large extent on the accuracy of phenotyping. Although enhanced yield of economically important product of a crop is the ultimate target in crop breeding, yield as a trait is highly complex to be deciphered in terms of effects of a gene or its allelic forms. Furthermore, association analysis attempts to relate a product of expression to an allele. Therefore, accurate estimation of gene effects is basic to the success of the approach. Association analysis requires accurate "phenotyping" of genotypes on a scale larger than any breeding-related analysis; hence, an approach such as "phenomics" is essential for unbiased estimation of effects of genes [198]. State-of-the-art phenomics facilities such as the Australian Plant Phenomics Facility with the Plant Accelerator involving the Waite Campus of the University of Adelaide and the High Resolution Plant Phenomics Centre involving CSIRO Plant Industry and the Australian National University in Canberra have highlighted the scientific advantages of automation in large-scale phenotyping in crop modeling. Such facilities include infrared and RGB imaging and other facilities to estimate total biomass, canopy temperature, and other features that are useful in evaluating a plant response to environmental stress and impulses. Since the procedures involved are nondestructive in nature, there is an added advantage for carrying forward the genotypes selected.

Besides, molecular dissection of tolerance mechanisms in heterologous systems that is an integral part of such approaches may lead to isolation of novel genes and

promoters. These genes can be used to improve tolerance in cultivated species or agronomically superior cultivars of sesame. Several genes for abiotic stress tolerance such as *codA*, *metD*, *tps1*, *mdh*, *hsp*(*s*), *acdS*, and sigma factors and antioxidant enzymes have been identified and utilized for development of transgenic crop plants. Isolation of such genes and alleles from indigenous resources will enrich the gene pool, which can further enhance both the transgenic and the conventional crop breeding programs. Phenomics approach also enables us to understand the precise molecular mechanism involved in conferring tolerance against different kinds of abiotic stresses.

Breeders conventionally go for early selection for characters such as biotic stresses, desirable plant type, and other yield-related traits. It is only at the advanced testing stages that the entries are tested for abiotic stresses when their numbers have been significantly reduced and are far from a complete representation of the initial material. Therefore, the success for breeding abiotic stress-resistant genotypes has been poor due to low selection intensity at this stage. The various reasons for having apprehensions on selection at early stages range from the presence of fewer differences between entries leading to reduction in the selection gain due to low heritabilities and variance for yield traits under abiotic stresses as the yields fall. Also, there are practical problems in selecting the best germplasm due to experiment-to-experiment variation because of high genotype × environment interaction in stress experiments. In addition, a general assumption that the high-yield entries in the stress-free environments will have the increased grain yield potential under the abiotic stress condition prevails. Finally but perhaps most importantly, lack of interest on the part of private seed sector for the resource-poor farmers due to their commercial interest in favor of rich farmers prevails that in turn unfortunately influences the public sector scientists also.

In addition to the constraints due to conventional breeding practices, the genetic considerations for the lack of abiotic stress tolerance in the cultivated gene pool cannot be ignored. Foremost among the genetic reasons is the founder effect that results in the genetic bottleneck during domestication of the crop plants. Occurrence of wild relatives as natural populations under the harsh environmental conditions in case of most of the crop plants confirms the loss of useful alleles during domestication.

Though the traditional/conventional approaches of plant breeding have contributed significantly to the increase in the crop productivity resulting in cultivars having higher tolerance to biotic stresses and better plant types, there is need to adopt modern biotechnological approaches for dealing with abiotic stresses as the success stories in this aspect are rather few. The basic causes responsible for this shift are [195] as follows:

1) Focus has been on yield rather than on specific traits.
2) Difficulties in breeding for tolerance traits as they are subjected to GXE interactions.
3) Relatively infrequent use of simple physiological traits as measures of tolerance.
4) Desired traits can be used only from closely related taxa.

48.8 Transgenic Approach

Transgenic approaches being employed for the transfer of abiotic stress tolerance to other crops could be helpful in sesame. The use of transgenic approaches for incorporation of useful genes often depends upon the efficient *in vitro* regeneration protocols. However, a highly efficient regeneration protocol for sesame has yet to be optimized. Several attempts have been made to develop efficient *in vitro* regeneration and transformation protocols for sesame in the past decades. The first initiative was taken by Govil and Singh [199] for haploid production using anthers. Whole plants were obtained by shoot tip culture by Lee *et al.* [200]. Further studies using shoot tip as explants resulted in callus [201], while multiple shoot buds to single plantlet were obtained when shoot tips used were pretreated [202] or were supplemented with kinetin in addition to BA [203]. Leaf segments as explants showed responses such as adventitious shoot formation and rooting of shoots [204]. Using anther as explant caused callus generation in most cases [205]. The use of hypocotyls and cotyledon gave no callus even under various combinations of media [201], while Lee *et al.* [206] were successful in attaining the callus stage. A different attempt via protoplast culture of hypocotyls also resulted in callus production [207]. Although, embryo-like structures and adventitious shoots were observed when reducing the concentration of both NAA and BA from the earlier adopted ones [208, 209]. In addition, direct usage of callus in protoplast culture also failed to generate plantlets and terminated at callusing [210]. Rooting of the regenerated shoots also poses problem. Some success was achieved by Takebayashi *et al.* [211], but the use of seedling or seed resulted only in callus formation [212] and subsequent variations of explants as wounded cotyledon lamina or deembryonate cotyledon as a whole ended up with multiple shoots [213, 214]. There is no report of standardized protocol for indirect regeneration of plants from callus. Xu *et al.* [215] reported a low plant conversion rate from somatic embyos that was followed by the report of induction of multiple shoots from nodal segments with axillary buds [216]. Further progress in shoot regeneration has been achieved at a maximum frequency of 63% and 4.4 shoots per regenerating explants [213]. Only recently, internodal thin-cell layer culture was reported to produce efficient shoots with rooting and establishment of 80% plantlets [217].

Success stories of *Agrobacterium*-mediated genetic transformation in sesame are few. Ogasawara *et al.* [218] transformed sesame for increasing the yield of naphthoquinone by the means of *Agrobacterium rhizogens* ATCC 15834 mediated hairy root culture. The use of *A. tumefaciens* by Taskin *et al.* [219] for expression of carrot calmodulin gene in another species of sesame (*S. schinzianum*) has been reported [220]. Production of recombinant proteins (a fungal protein, phytase) has been achieved by sesame hairy root cultures [221]. Recently, successful recovery of fertile transgenic plants was achieved using *A. tumaefaciens* strain with cotyledon explants that displayed a transformation efficiency of 1.01% [222]. In view of the above, any attempt to use transgenic technologies for incorporating abiotic stress in sesame should prefer methods where the *in vitro* regeneration step is not needed. This includes floral dip method where flowering shoots are swirled in a solution of

Agrobacterium, resulting in the formation of transgenic seeds [223, 224]. Of the various biolistic methods that involve physical DNA delivery, microprojectile bombardment of tissues has also proven useful for transforming plants that lack good regeneration systems [225]. Another method involving infection of apical meristem of the differentiated embryo of the germinating seedling with *Agrobacterium* has worked well in the recalcitrant cotton resulting in transgenic T1 plants [226]. However, in groundnuts infection of *Agrobacterium* after pricking of embryo axes of mature seeds with one excised cotyledon directly gave transformed seedlings [227]. Nevertheless, lack of protocols necessary for transgenic development in sesame may get compensated with extremely high genetic variability present in the cultivated gene pool of sesame and its wild allies [5].

High-throughput technologies along with powerful bioinformatics tools are proving useful to meet the challenges in understanding the abiotic stress tolerance in more than one way. The "omics" approaches have opened up the genome, its transcriptional products, regulatory networks, signaling pathways and their interactions, and the metabolites involved in the entire gamut of stress tolerance spanning from its perception, differential gene expression, and imparting protection from damage caused due to abiotic stress. Therefore, the functional genomics approaches involving transcriptome, interactome, and metabolome profiling of the plant subjected to specific stress environment are required in sesame (Table 48.1).

Table 48.1 The generalized key steps, activities, and expected outputs involved during incorporation of abiotic stress tolerance.

Key steps	What needs to be done	Output
Identification of trait donor	Large-scale germplasm screening including wild relatives	Core/reference collection for fine scale analysis
	Phenomics for fine dissection of underlying mechanism	Donors for specific mechanisms of tolerance
	Functional genomics	Understanding the natural mechanisms and pathways
	Association genetics	SNPs for specific traits
	Genome-wide approach	Allelic variations for specific mechanisms
	Candidate gene approach	Marker tags for specific traits
	Allele mining	Superior/elite germplasm for direct utilization
	Gene mapping and marker tagging	
Understanding the mechanism of tolerance	Functional genomics	Identification of genes/pathways/networks for tolerance
	Systems biology approach	
Incorporation of the stress tolerance	Conventional back cross breeding	Abiotic stress resistant varieties
	Marker assisted breeding	
	Transgenic approach	

Research efforts on incorporation of abiotic tolerance in sesame can greatly benefit from the lessons learnt from other crops where considerable progress has already been made. The studies on large number of crops indicate that pathways and gene networks between different abiotic stresses overlap significantly. On the other hand, managing tolerance to one type of stress can result in making the plants susceptible to other type of stress due to opposing physiological and molecular processes. Thorough investigations into the individual stress response may contribute to the understanding of the basic mechanisms involved, but would be largely unsuccessful in translating this knowledge for developing genotypes that exhibit superior performance in the field. Therefore, there is need to study the multiple stresses simultaneously in various combinations. In addition, due consideration needs to be given to the phenology-related parameters of the plant also as similar stresses are known to affect plants with different life cycle differently.

Research efforts and funds to "orphan" crops such as sesame that go beyond the mere increase in crop yields deserve special emphasis as they are crucial for food and nutritional security on a regional or local basis. Though gross economic and welfare impacts of investments made in crops such as sesame may appear to give low returns, the alternative indicators such as promotion of crop and genetic diversity leading to overall agricultural stability highlight the urgent need to pay attention to these crops [228]. In addition, an understanding of the basic mechanisms of the unique traits lacking in major plants and model systems such as extraordinary antioxidative ability of sesame seed oil, drought tolerance, ability to accumulate heavy metals, and so on can be utilized for the improvement of major cops subsequently.

References

1 Grover, A., Kapoor, A., Lakshmi, O.S. *et al.* (2001) *Curr. Sci.*, **80**, 206–216.
2 Boyer, J.S. (1982) *Science*, **218**, 443–448.
3 Bansal, K.C. (2004) Transgenic crops with enhanced tolerance to abiotic stresses, in *Biotechnological Approaches for Sustainable Development* (eds M.S. Reddy and S. Khanna), Allied Publishers Pvt. Ltd., New Delhi, India, pp. 58–64.
4 Bedigian, D. and Harlan, J.R. (1986) *Econ. Bot.*, **40**, 137–154.
5 Bhat, K.V., Babrekar, P.P., and Lakhanpaul, S. (1999) *Euphytica*, **110**, 21–33.
6 Ashri, A. (1988) Sesame breeding-objectives and approaches, in Oil Crops-Sunflower, Linseed and Sesame, Proc. 4th Oil Crop Network Workshop, Njoro, Kenya, IDRC-MR20e, IDRC.
7 Moazzami, A.A. and Eldin, A.K. (2006) *J. Am. Oil Chem. Soc.*, **83**, 719–723.
8 Ghafoorunissa (1996) *Lipids*, **31**, 287–291.
9 Abou-Gharbia, H.A., Shahidi, F., Shehata, A.A.Y., and Youssef, M.M. (1997) *J. Am. Oil Chem. Soc.*, **74**, 215–221.
10 Bedigian, D. (2003) *Genet. Resour. Crop. Evol.*, **50**, 779–787.
11 Beare-Rogers, J., Ghafoorunissa, K.O., Gérard, R. *et al.* (1998) *Food Nutr. Bull.*, **19**, 250–266.
12 Abitogun, A.S., Jide, A.O., Arawande, J.O. *et al.* (2009) *Internet J. Nutr. Wellness*, **8**, 2.
13 Cagirgan, M.I. (2006) *Field Crops Res.*, **96**, 19–24.
14 Ashri, A. (1989) Sesame, in *Oil Crops of the World* (eds G. Roebblen, R.K. Downey, and A. Ashri), McGraw Hill Publishing Co., New York, pp. 375–387.

15 Duhoon, S.S., Koppar, H.N., and Srivastava, U.C. (2002) *Nat. J. Plant Improve.*, **2**, 32–39.

16 Cagirgan, M.I. (2001) Mutation techniques in sesame (*Sesamum indicum* L.) for intensive management: confirmed mutants, in *Sesame Improvement by Induced Mutations*, IAEA, Vienna, IAEA-TECDOC-1195, pp. 31–40.

17 Ucan, K., Killi, F., Gencoglan, C., and Merdun, H. (2007) *Field Crops Res.*, **101**, 249–258.

18 Langham, D.R. and Wiemers, T. (2001) Progress in mechanizing sesame in the US through breeding, in *Trends in New Crops and New Uses* (eds J. Janick and A. Whipkey), ASHS Press, Alexandria, VA, pp. 157–173.

19 Christianson, J.A., Llewellyn, D.J., Dennis, E.S., and Wilson, I.W. (2010) *Plant Cell Physiol.*, **51**, 21–37.

20 Perata, P. and Voesenek, A.C.J. (2006) *Trends Plant Sci.*, **12**, 43–46.

21 Dennis, E.S., Dolferus, R., Ellis, M. *et al.* (2000) *J. Exp. Bot.*, **51**, 89–97.

22 Klok, E.J., Wilson, I.W., Wilson, D. *et al.* (2002) *Plant Cell*, **14**, 2481–2494.

23 Levitt, J. (1980) Responses of plants to environmental stress, in *Chilling, Freezing, and High Temperature Stress*, Academic Press, New York, vol. 1.

24 Oplinger, E.S., Putnam, D.H., Kaminski, A.R. *et al.* (1990) Alternative Food Crops Manual Sesame University of Wisconsin-Extension, University of Minnesota, Center for Alternative Plant and Animal Products and the Minnesota Extension Service.

25 Beroza, M. and Kinman, M.L. (1955) *J. Am. Oil Chem. Soc.*, **32**, 348–350.

26 Gong, Z., Lee, H., Xiong, L. *et al.* (2002) *Proc. Natl. Sci. Acad. USA*, **99**, 11507–11512.

27 Dong, C.H., Hu, X., Tang, W. *et al.* (2006) *Mol. Cell Biol.*, **26**, 9533–9543.

28 Winfield, M.O., Lu, C., Wilson, I.D. *et al.* (2010) *Plant Biotech. J.*, **8**, 749–771.

29 Fowler, S. and Thomashow, M.F. (2002) *Plant Cell*, **14**, 1675–1690.

30 Park, E.J., Jeknic, Z., and Chen, T.H.H. (2006) *Plant Cell Physiol.*, **47**, 706–714.

31 Kodama, H., Hamada, T., Horiguchi, G. *et al.* (1994) *Plant Physiol.*, **105**, 601–605.

32 Kim, J.C., Lee, S.H., Cheong, Y.H. *et al.* (2001) *Plant J.*, **25**, 247–259.

33 Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G. *et al.* (1998) *Science*, **280**, 104–106.

34 Tamminen, I., Makela, P., Heino, P., and Palva, E.T. (2001) *Plant J.*, **25**, 1–8.

35 Yuanyuan, M., Yali, Z., Jiang, L., and Hongbo, S. (2009) *Afr. J. Biotechnol.*, **8**, 2004–2010.

36 Gilmour, S.J., Sebolt, A.M., Salazar, M.P. *et al.* (2000) *Plant Physiol.*, **124**, 1854–1865.

37 Kasuga, M., Liu, Q., Miura, S. *et al.* (1999) *Nature Biotech.*, **17**, 287–291.

38 Griffith, M. and Yaish, M.W. (2004) *Trends Plant Sci.*, **9**, 399–405.

39 Fan, Y., Liu, B., Wang, H. *et al.* (2002) *Plant Cell Rep.*, **21**, 296–301.

40 Zhu, J., Dong, C.H., and Zhu, J.K. (2007) *Curr. Opin. Plant Biol.*, **10**, 290–295.

41 Yahya, A. (1998) Responses to soil salinity of sesame (*Sesamum indicum* L.) and sugar beet (*Beta vulgaris* L.). Doctoral Thesis, Uppsala.

42 Koca, H., Bor, M., Ozdemir, F., and Turkan, I. (2007) *Environ. Exp. Bot.*, **60**, 344–351.

43 Greenway, H. and Munns, R. (1980) *Annu. Rev. Plant Physiol.*, **31**, 149–190.

44 Matysik, J., Alia, B.B., and Mohanty, P. (2002) *Curr. Sci.*, **82**, 525–532.

45 Gzik, A. (1996) *Environ. Exp. Bot.*, **36**, 29–38.

46 Jain, S., Nainawatee, H.S., Jain, R.K., and Chowdhury, J.B. (1991) *Plant Cell Rep.*, **9**, 684–687.

47 Petrusa, L. and Winicov, I. (1997) *Plant Physiol. Biochem.*, **35**, 303–310.

48 Gossett, D.G., Millhollon, E.P., and Lucas, M.C. (1994) *Crop. Sci.*, **34**, 706–714.

49 Hernandez, J.A., Jimenez, A., Mullineaux, P. *et al.* (2000) *Plant Cell Environ.*, **23**, 853–862.

50 Benavides, M.P., Marconi, P.L., Gallego, S.M. *et al.* (2000) *Aust. J. Plant. Physiol.*, **27**, 273–278.

51 Mittova, V., Tal, M.V.M., and Guy, M. (2002) *Physiol. Plant.*, **115**, 393–400.

52 Bor, M., Ozdemir, F., and Turkan, I. (2003) *Plant Sci.*, **164**, 77–84.

53 Gehlot, H.S., Purohit, A., and Shekhawat, N.S. (2005) *J. Cell Mol. Biol.*, **4**, 31–39.

54 Loewus, F.A., Everand, J.D., and Young, K.A. (1990) Inositol metabolism: precursor role and breakdown, in *Inositol Metabolism in Plants* (eds D.J. Morre, W.F. Boss, and F.A. Loewus), Wiley-Liss, New York, pp. 21–45.

55 Yoshida, K.T., Wada, T., Koyama, H. *et al.* (1999) *Plant Physiol.*, **119**, 65–72.

56 Loewus, F.A. and Murthy, P.P.N. (2000) *Plant Sci.*, **150**, 1–19.

57 Nelson, D.E., Rammesmayer, G., and Bohnert, H.J. (1998) *Plant Cell*, **10**, 753–764.

58 Nelson, D.E., Koukoumanos, M., and Bohnert, H.J. (1999) *Plant Physiol.*, **119**, 165–172.

59 Ishitani, M., Majumder, A.L., Bornhouser, A. *et al.* (1996) *Plant J.*, **9**, 537–548.

60 Fernandez, P., Rienzo, J.D., Fernandez, L. *et al.* (2008) *BMC Plant Biol.*, **8**, 11.

61 Ramani, S. and Apte, S.K. (1997) *Biochem. Biophys. Res. Commun.*, **233**, 663–667.

62 Tsiantis, M.S., Bartholomew, D.M., and Smith, J.A.C. (1996) *Plant J.*, **9**, 729–736.

63 Zhu, J.K., Hasegawa, P.M., and Bressan, R.A. (1997) *Crit. Rev. Plant Sci.*, **16**, 253–277.

64 Chauhan, S., Forsthoefel, N., Ran, Y. *et al.* (2000) *Plant J.*, **24**, 511–522.

65 Wei, J.Z., Tirajoh, A., Effendy, J., and Plant, A. (2000) *Plant Sci.*, **159**, 135–148.

66 Kuol, B., Janssens, M.J.J., Abdalla, A., and Tropentag, D. (2003) Technological and institutional innovations for sustainable rural development, Conference on International Agricultural Research for Development, Gottingen.

67 Kim, K.S., Park, S.H., and Jenks, M.A. (2006) *J. Plant Physiol.*, **164**, 1134–1143.

68 Hassanzadeh, M., Ebadi, A., Panahyan-e-Kivi, M. *et al.* (2009) *Res. J. Environ. Sci.*, **3**, 239–244.

69 Fazeli, F., Ghorbanli, M., and Niknam, V. (2007) *Biol. Plant.*, **51**, 98–103.

70 Kosma, D.K., Bourdenx, B., Bernard, A. *et al.* (2009) *Plant Physiol.*, **151**, 1918–1929.

71 Kannangara, R., Branigan, C., Liu, Y. *et al.* (2007) *Plant Cell*, **19**, 1278–1294.

72 Shahandeh, H. and Hossner, L.R. (2000) *Int. J. Phytoremediation*, **2**, 269–286.

73 Angelova, V., Ivanova, R., and Ivanov, K. (2004) *Commun. Soil Sci. Plant Anal.*, **35**, 2551–2566.

74 Gupta, A.K. and Sinha, S. (2006) *Chemosphere*, **64**, 161–173.

75 Abdel-Sabour, M.F. (2007) *EJEAFChe*, **6**, 2178–2198.

76 Abdel-Sabour, M.F. and Abo El-Seoud, M.A. (1996) *Agric. Ecosyst. Environ.*, **60**, 157–164.

77 Bharti, N. and Singh, R. (1993) *Phytochemistry*, **33**, 531–534.

78 Kader, J.C. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 627–654.

79 Bession, F., Koo, A.J.K., Ruuska, S. *et al.* (2003) *Plant Physiol.*, **132**, 681–697.

80 Kim, T.H., Kim, M.C., Park, J.H. *et al.* (2006) *J. Plant Biol.*, **49**, 371–375.

81 Molina, A., Diaz, I., Vasil, I.K. *et al.* (1996) *Mol. Gen. Genet.*, **252**, 162–168.

82 Trevino, M.B. and O'Connell, M.A. (1998) *Plant Physiol.*, **116**, 1461–1468.

83 Vignols, F., Wigger, M., Garcia-Garrido, J.M. *et al.* (1997) *Gene*, **195**, 177–186.

84 Yubero-Serrano, E.M., Moyano, E., Medina-Escobar, N. *et al.* (2003) *J. Exp. Bot.*, **54**, 1865–1877.

85 Ouvrard, O., Cellier, F., Ferrare, K. *et al.* (1996) *Plant Mol. Biol.*, **31**, 819–829.

86 Jiang, Y. and Deyholos, M.K. (2006) *BMC Plant Biol.*, **6**, 25.

87 Torres-Schumann, S., Godoy, J., and Pintor-Toro, J. (1992) *Plant Mol. Biol.*, **18**, 749–757.

88 Holmberg, N. and Bulow, L. (1998) *Trends Plant Sci.*, **3**, 61–66.

89 Nieuwland, J., Feron, R., Huisman, B. *et al.* (2005) *Plant Cell*, **7**, 2007–2019.

90 Canevascini, S., Caderas, D., Mandel, T. *et al.* (1996) *Plant Physiol.*, **112**, 513–524.

91 Thoma, S., Hecht, U., Kippers, A. *et al.* (1994) *Plant Physiol.*, **105**, 35–36.

92 Pyee, J. and Kolattukudy, P.E. (1995) *Plant J.*, **7**, 49–59.

93 Carvalho, A.O., Teodoro, C.E.S., Da Cunha, M. *et al.* (2004) *Physiol. Plant.*, **122**, 328–336.
94 Tsuboi, S., Osafune, T., Tsugek, I.R. *et al.* (1992) *J. Biochem.*, **111**, 500–508.
95 Choi, A.M., Lee, S.B., Cho, S.H. *et al.* (2008) *Plant Physiol. Biochem.*, **46**, 127–139.
96 Naested, H., Frandsen, G.I., Jauh, G.Y. *et al.* (2000) *Plant Mol. Biol.*, **44**, 436–476.
97 Chen, E.C.F., Tai, S.S.K., Peng, C.C., and Tzen, J.T.C. (1998) *Plant Cell Physiol.*, **39**, 935–941.
98 Frandsen, G.I., Muller-Uri, F., Nielsen, M. *et al.* (1996) *J. Biol. Chem.*, **271**, 343–348.
99 Takahashi, S., Katagiri, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2000) *Plant Cell Physiol.*, **41**, 898–903.
100 Chen, J.C.F., Tsai, C.C.Y., and Tzen, J.T.C. (1999) *Plant Cell Physiol.*, **40**, 1079–1086.
101 Hatzopoulos, P., Franz, G., Choy, L., and Sung, R.Z. (1990) *Plant Cell*, **2**, 457–467.
102 Seki, M., Narusaka, M., and Abe, H. (2001) *Plant Cell*, **13**, 61–72.
103 Magnan, F., Ranty, B., Charpenteau, M. *et al.* (2008) *Plant J.*, **56**, 575–589.
104 Alexandre, C., Moller-Steinbach, Y., Schonrock, N. *et al.* (2009) *Mol. Plant*, **2**, 675–687.
105 Abe, H., Urao, T., Ito, T. *et al.* (2003) *Plant Cell*, **15**, 63–78.
106 Hanano, A., Burcklen, M., and Flenet, M. (2006) *J. Biol.Chem.*, **281**, 33140–33151.
107 Lequeu, J., Fauconnier, M., Chammai, A. *et al.* (2003) *Plant J.*, **36**, 155–184.
108 Aubert, Y., Vile, D., Pervent, M. *et al.* (2010) *Plant Cell Physiol.*, **51**, 1975–1987.
109 Hsiao, E.S.L. and Tzen, J.T.C. (2011) *Plant Physiol. Biochem.*, **49**, 77–81.
110 Chinnusamy, V., Gong, Z., and Zhu, J.K. (2008) *J. Integr. Plant Biol.*, **50**, 1187–1195.
111 Lin, L.J., Tai, S.S.K., Peng, C.C., and Tzen, J.T.C. (2002) *Plant Physiol.*, **128**, 1200–1211.
112 Bray, E. (2002) *Ann. Bot.*, **89**, 803–811.
113 Giacomelli, L., Rudella, A., and Wijk, K.J. (2006) *Plant Physiol.*, **141**, 685–701.
114 Li, F., Asami, T., Wu, X. *et al.* (2007) *Plant Physiol.*, **145**, 87–97.
115 Bateman, A., Birney, E., Cerruti, L. *et al.* (2002) *Nucl. Acids Res.*, **30**, 276–280.
116 Turk, B., Turk, V., and Turk, D. (1997) *Biol. Chem.*, **378**, 141–150.
117 Valdes-Rodrıguez, S., Guerrero-Rangel, A., Melgoza-Villagomez, C. *et al.* (2007) *Plant Physiol. Biochem.*, **45**, 790–798.
118 Hwang, J.E., Hong, J.K., Lim, C.J. *et al.* (2010) *Plant Cell Rep.*, **29**, 905–915.
119 Gaddour, K., Vicente-Carbajosa, J., Lara, P. *et al.* (2001) *Plant Mol. Biol.*, **45**, 599–608.
120 Pernas, M., Sanchez-Monge, R., and Salcedo, G. (2000) *FEBS Lett.*, **467**, 206–210.
121 Shyu, D.J.H., Chou, W.M., Yiu, T.J. *et al.* (2004) *J. Agric. Food Chem.*, **52**, 1350–1356.
122 Peng, C.C., Shyu, D.J.H., Chou, W.M. *et al.* (2004) *J. Agric. Food Chem.*, **52**, 3115–3119.
123 Dhindsa, R.S. and Matowe, W. (1981) *J. Exp. Bot.*, **32**, 79–91.
124 Spychalla, J.P. and Desborough, S.L. (1990) *Plant Physiol.*, **94**, 1214–1218.
125 Parida, A.K. and Das, A.B. (2005) *Ecotoxicol. Environ.*, **60**, 324–349.
126 Moazzami, A. (2006) Sesame seed lignans. Doctoral dissertation. Department of Food Science, SLU.
127 Namiki, M. (1995) *Food Rev. Intern.*, **11**, 281–329.
128 Tashiroa, T., Fukudab, Y., Osawaa, T., and Namikia, M. (1990) *J. Am. Oil Chem. Soc.*, **67**, 508–511.
129 Hemalatha, S. and Ghafoorunissa (2004) *J. Am. Oil Chem. Soc.*, **81**, 467–470.
130 Fukuda, Y. and Namiki, M. (1988) *Nippon Shokuhin Kogyo Gakkaishi*, **35**, 552–562.
131 Ryu, S.N. (2010) *Korean J. Intl. Agri.*, **22**, 50–53.
132 Sadeghi, N., Oveisi, M.R., Hajimahmoodi, M. *et al.* (2009) *Iranian J. Pharma. Res.*, **8**, 101–105.
133 Shirato-Yasumoto, S., Komeichi, M., Okuyama, Y., and Horigane, A. (2003) *SABRAO J. Breed. Genet.*, **35**, 27–34.
134 Dachtler, M., Frans, H.M., Frans, S. *et al.* (2003) *Eur. J. Lipid Sci. Technol.*, **105**, 488–496.

135 Kang, M.H., Naito, M., Sakai, K. *et al.* (2000) *Life Sci.*, **66**, 161–171.

136 Ono, E., Nakai, M., Fukui, Y. *et al.* (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 10116–10121.

137 Suh, M.C., Kim, M.J., Hur, C.G. *et al.* (2003) *Plant Mol. Biol.*, **52**, 1107–1123.

138 Tsuruoka, N., Kidokoro, A., Matsumoto, I. *et al.* (2005) *Biosci. Biotechnol. Biochem.*, **69**, 179–188.

139 Shimizu, S., Akimoto, K., Kawashima, H. *et al.* (1991) *Lipids*, **26**, 512–516.

140 Hirose, N., Inoue, T., Nishihara, K. *et al.* (1991) *J. Lipid Res.*, **32**, 629–638.

141 Sawada, R.U., Fujiwara, Y., and Igarashi, O. (1994) *Biosci. Biotechnol. Biochem.*, **58**, 2114–2115.

142 Hess, J.L. (1993) Vitamin E, α-tocopherol, in *Antioxidants in Higher Plants* (eds R.G. Alscher and J.L. Hess), CRC Press, Boca Raton, FL, pp. 111–134.

143 Cooney, R.V., Custer, L.J., Okinaka, L., and Franke, A.A. (2001) *Nutr. Cancer*, **39**, 66–71.

144 Hirschberg, J. (1999) *Curr. Opin. Biotechnol.*, **10**, 186–191.

145 Tanaka, R.U., Oster, E., Rudiger, K.W., and Grimm, B. (1999) *Plant Physiol.*, **120**, 695–704.

146 Park, M.R., Cho, E.A., Rehman, S., and Yun, S.J. (2010) *Pakistan J. Bot.*, **42**, 1815–1825.

147 Keegstra, K. and Cline, K. (1999) *Plant Cell*, **11**, 557–570.

148 Keegstra, K., Olsen, L.J., and Theg, S.M. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **40**, 471–501.

149 Kleiger, G. and Eisenberg, D. (2002) *J. Mol. Biol.*, **323**, 69–76.

150 Uno, Y., Furihata, T., Abe, H. *et al.* (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 11632–11637.

151 Yoshida, R., Hobo, T., Ichimura, K. *et al.* (2002) *Plant Cell Physiol.*, **43**, 1473–1483.

152 Giannino, D., Condello, E., Bruno, L. *et al.* (2004) *J. Exp. Bot.*, **55**, 2063–2073.

153 Keller, Y., Bouvier, F.D., and Harlingue, A. (1988) *Eur. J. Biochem.*, **251**, 413–417.

154 Mayer, R.R., Cherry, J.L., and Rhodes, D. (1990) *Phytochemistry*, **94**, 796–810.

155 Serraj, R., Shelp, B.J., and Sinclair, T.R. (1998) *Physiol. Plant.*, **102**, 79–86.

156 Mazzucotelli, E., Tartari, A., Cattivelli, L., and Forlani, G. (2006) *J. Exp. Bot.*, **57**, 3755–3766.

157 Su, G.X., Yu, B.J., Zhang, W.H., and Liu, Y.L. (2007) *Plant Physiol. Biochem.*, **45**, 560–566.

158 Bor, M., Seckin, B., Ozgur, R. *et al.* (2009) *Acta Physiol. Plant.*, **31**, 655–659.

159 Guo, P., Baum, M., Grando, S. *et al.* (2009) *J. Exp. Bot.*, **60**, 3531–3544.

160 Abebe, T., Melmaiee, K., Berg, V., and Wise, R.P. (2010) *Funct. Integr. Genomics*, **10**, 191–205.

161 Song, H., Xu, X., Wang, H. *et al.* (2010) *J. Sci. Food Agric.*, **90**, 1410–1416.

162 Fait, A., Fromm, H., Walter, D. *et al.* (2008) *Trends Plant Sci.*, **13**, 14–19.

163 Bouche, N., Yellin, A., Snedden, W.A., and Fromm, H. (2005) *Annu. Rev. Plant Biol.*, **56**, 435–466.

164 Robinson, N.J., Tommey, A.M., Kuske, C., and Jackson, P.J. (1993) *Biochem. J.*, **295**, 1–10.

165 Cobbett, C. and Goldsbrough, P. (2002) *Annu. Rev. Plant Biol.*, **53**, 159–182.

166 Hudspeth, R.L., Hobbs, S.L., Anderson, D.M. *et al.* (1996) *Plant Mol. Biol.*, **31**, 701–705.

167 Zhou, J. and Goldsbrough, P.B. (1994) *Plant Cell*, **6**, 875–884.

168 Hsieh, H.M., Liu, W.K., and Huang, P.C. (1995) *Plant Mol. Biol.*, **28**, 381–389.

169 Guo, W.J., Bundithya, W., and Goldsbrough, P.B. (2003) *New Phytol.*, **159**, 369–381.

170 Lane, B.G., Kajioka, R., and Kennedy, T.D. (1987) *Biochem. Cell Biol.*, **65**, 1001–1005.

171 Chyan, C.L., Lee, T.T., Liu, C.P. *et al.* (2005) *Biosci. Biotechnol. Biochem.*, **69**, 2319–2325.

172 Robinson, N.J., Wilson, J.R., and Turner, J.S. (1996) *Plant Mol. Biol.*, **30**, 1169–1179.

173 Roosens, N.H., Bernard, C., Leplae, R., and Verbruggen, N. (2004) *FEBS Lett.*, **577**, 9–16.

174 Lee, J., Shim, D., Song, W.Y. *et al.* (2004) *Plant Mol. Biol.*, **54**, 805–815.

175 Merrifield, M.E., Ngu, T., and Stillman, M.J. (2004) *Biochem. Biophys. Res. Commun.*, **324**, 127–132.

176 Zimeri, A.M., Dhankher, O.P., McCaig, B., and Meagher, R.B. (2005) *Plant Mol. Biol.*, **58**, 839–855.

177 Jin, S., Cheng, Y., Guan, Q. *et al.* (2006) *Biotechnol. Lett.*, **28**, 1749–1753.

178 Wisniewski, M., Bassett, C., Norelli, J. *et al.* (2008) *Physiol. Plant.*, **133**, 298–317.

179 Miller, J.D., Arteca, R.N., and Pell, E.J. (1999) *Plant Physiol.*, **120**, 1015–1024.

180 Akashi, K., Nishimura, N., Ishida, Y., and Yokota, A. (2004) *Biochem. Biophys. Res. Commun.*, **323**, 72–78.

181 Brkljacic, J.M., Samardzic, J.T., Timotijevic, G.S., and Maksimovic, V.R. (2004) *J. Plant Physiol.*, **161**, 741–746.

182 Wong, H.L., Sakamoto, T., Kawasaki, T. *et al.* (2004) *Plant Physiol.*, **135**, 1447–1456.

183 Yang, J., Wang, Y., Liu, G. *et al.* (2011) *Mol. Biol. Rep.*, **38**, 1567–1574.

184 Yang, Z., Wu, Y., Ye, L. *et al.* (2009) *Plant Mol. Biol.*, **70**, 219–229.

185 Xue, T.T., Li, X.Z., Zhu, W. *et al.* (2009) *J. Exp. Bot.*, **60**, 339–349.

186 Koh, M. and Kim, H.J. (2001) *Bull. Korean Chem. Soc.*, **22**, 362–366.

187 Thangavelu, S. (1994) Diversity in wild and cultivated species of Sesamum and its use, in *Sesame Biodiversity in Asia. Conservation. Evaluation and Improvement* (eds R.K. Arora and K.W. Riley), International Plant Genetic Resources Institute, New Delhi, India, pp. 13–23.

188 Ashri, A. (1985) Sesame improvement by large-scale cultivars intercrossing and by crosses with indehiscent and determinate lines, in *Sesame and Safflower: Status and Potentials* (ed. A. Ashri), FAO Plant Production and Protection Paper, Rome, 66, pp. 177–181.

189 Ashri, A. (1987) Report on FAO/IAEA expert consultation on breeding improved sesame cultivars. Crop Production and Protection Division, FAO Rome and Joint FAO/IAEA Division, Vienna. p. 14.

190 Rajan, S.S. (1981) Sesame breeding material and methods, in *Sesame: Status and Improvement* (ed. A. Ashri), FAO Plant Production and Protection Paper, Rome, 29, pp. 97–102.

191 Sharma, S.M. (1984) Constraints and opportunities for increasing the productivity and production of sesame in India in Proc. of Oilseed Production and Utilization. Constraints and Opportunities (eds H.C. Srivastava, S. Bhaskaran, B. Vatsya, and K.K.G. Menon), New Delhi, India, pp. 165–179.

192 Sharma, S.M. (1985) Sesamum research and its progress in India, in *Oil Crop: Sesame and Safflower* (ed. A. Omran), IDRC, Ottawa, pp. 11–27.

193 Duhoon, S.S., Sharma, S.M., Lakhanpaul, S., and Bhat, K.V. (2004) Sesame, in *Plant Genetic Resources: Oilseed and Cash Crops* (eds B.S. Dhillon, R.K. Tyagi, S. Saxena, and A. Agrawal) Narosa Publication House, New Delhi, India, pp. 118–135.

194 Ishitani, M., Rao, I., Wenzl, P. *et al.* (2004) *Field Crops Res.*, **90**, 35–45.

195 Tester, M. and Bacic, A. (2005) *Plant Physiol.*, **137**, 791–793.

196 Buckler, E.S., Gore, M., Zhu, C., and Yu, J. (2009) *Plant Genome*, **1**, 5–20.

197 Bisht, I.S., Mahajan, R.K., Loknathan, T.R., and Agarwal, R.C. (1998) *Genet. Resour. Crop. Evol.*, **45**, 325–335.

198 Houle, D., Diddahally, R., Govindaraju, D.R., and Omholt, S. (2010) *Nature Rev. Genet.*, **11**, 855–866.

199 Govil, C.M. and Singh, V.R.R. (1982) in Proceedings of the 5th International Congress Plant Tissue and Cell Culture, pp. 545–546.

200 Lee, J.I., Park, Y.H., Park, Y.S. *et al.* (1985) *Korean J. Breed.*, **17**, 367–372.

201 George, L., Bapat, V.A., and Rao, P.S. (1987) *Ann. Bot.*, **60**, 17–21.

202 George, L., Bapat, V.A., and Rao, P.S. (1989) *Proc. Indian Acad. Sci. Plant Sci.*, **99**, 135–137.

203 Baskaran, P. and Jayabalan, N. (2006) *J. Agri. Technol.*, **2**, 259–269.

204 Datta, A.K. and Biswas, A.K. (1986) *Cell Chromosome Res.*, **9**, 7–10.

205 Ranaweera, K.K.D.S. and Pathirana, R. (1992) *J. Natl. Sci. Council Sri Lanka*, **20**, 309–315.

206 Lee, S.Y., Kim, H.S., and Lee, Y.T. (1988) *Res. Rep. Rural Ev. Dm.*, **30**, 69–73.

207 Shoji, K., Masuda, K., Sugai, M. *et al.* (1988) *Cytologia*, **53**, 205–211.

208 Masuda, K. (1989) Organogenesis in sesame tissue culture, in *Sesame Science* (eds. M. Namiki and T. Kobayashi), Asakura Shoten, Tokyo, pp. 73–81.

209 Kwon, T.H., Abe, T., and Sasahara, T. (1993) *Plant Tissue Cult.*, **10**, 260–266.

210 Bapat, V.A., George, L., and Rao, P.S. (1989) *Indian J. Exp. Biol.*, **27**, 82–184.

211 Takebayashi, K., Mimura, A., Ichikawa, A. *et al.* (1994) *Plant Tissue Cult. Lett.*, **1**, 129–133.

212 Saravanan, S. and Nadarajan, N. (2005) *Res. J. Agr. Biol. Sci.*, **1**, 98–100.

213 Were, B.A., Gudu, S., Onkware, A.O. *et al.* (2006) *Plant Cell Tissue Organ Cult.*, **85**, 235–239.

214 Seo, H.Y., Kim, Y.J., Park, T.I. *et al.* (2007) *In vitro Cell. Dev. Biol.*, **43**, 209–214.

215 Xu, Z.Q., Jia, J.F., and Hu, Z.D. (1997) *J. Plant Physiol.*, **150**, 755–758.

216 Gangopadhyay, G., Poddar, R., and Gupta, S. (1998) *Phytomorphology*, **48**, 83–90.

217 Chattopadhyaya, B., Banerjee, J., Basu, A. *et al.* (2010) *Plant Biotechnol. Rep.*, **4**, 173–178.

218 Ogasawara, T., Chiva, K., and Tada, M. (1993) *Phytochemistry*, **33**, 1095–1098.

219 Taskin, K.M., Ercan, A.G., and Turgut, K. (1999) *Turk. J. Bot.*, **23**, 291–295.

220 Mitsuma, S., Ishigaki, E., Sugiyama, R. *et al.* (2004) *Biol. Pharm. Bull.*, **10**, 1621–1625.

221 Jin, U.H., Chun, J.A., Han, M.O. *et al.* (2005) *Process Biochem.*, **40**, 3754–3762.

222 Yadav, M., Chaudhary, D., Sainger, M. *et al.* (2010) *Plant Cell Tissue Organ Cult.*, **103**, 377–386.

223 Bechtold, N., Ellis, J., and Pelletier, G. (1993) *C. R. Acad. Sci. Paris Life Sci.*, **316**, 1194–1199.

224 Bent, A.F. and Clough, S.J. (1998) Agrobacterium germ-line transformation: transformation of *Arabidopsis* without tissue culture, in *Plant Molecular Biology Manual* (ed. S.B. Gelvin), Kluwer Academic Publishers, the Netherlands, pp. 1–14.

225 Gupta, A.K., Anoop, N., Mushtaq, A., and Mathangi, V. (1999) *J. Punjab Acad. Sci.*, **1**, 27–34.

226 Keshamma, E., Rohini, S., Rao, K.S. *et al.* (2008) *J. Cott. Sci.*, **12**, 264–272.

227 Rohini, V.K. and Rao, K.S. (2000) *Plant Sci.*, **150**, 41–49.

228 Nelson, R.J., Naylor, R.L., and Jahn, M.M. (2004) *Crop Sci.*, **44**, 1901–1904.

49
Jatropha curcas: Approaches to Engineer Fatty Acid Synthesis and Abiotic Stress Tolerance

Nalini Eswaran and Tangirala Sudhakar Johnson

Owing to volatile crude oil prices, increased demand for transportation fuel in emerging economies, and changes in global climatic conditions, it is widely agreed that more sustainable alternative energy sources need to be developed in the near future. *Jatropha curcas*, a member of Euphorbiaceae, is emerging as the most promising tree-borne oil seed as a source of biodiesel as it does not compete with conventional food crops and has a lipid composition similar to that of fossil fuel. However, nonavailability of improved varieties and production under semiarid conditions marred the prospects of being a successful energy crop. In this review, we present the progress and genetic engineering approaches toward the improvement of two important traits, namely, fatty acid synthesis and abiotic stress tolerance. Integration of "omics" approach for identifying key regulatory elements, genes, with metabolic engineering is essential to realize the full potential of *Jatropha curcas* as an energy crop.

49.1
Introduction

Increase in crude oil prices, global warming, and limited reserves of fossil fuels underlined the importance of renewable and alternative energy sources such as biofuel. Among biofuels, bioethanol and biodiesel are considered the first-generation fuels, which are progressively contributing to the decrease in dependency on fossil fuel. The keen interest in biofuels is mainly driven both by climate change issues, aiming to reduce carbon emissions, and by geopolitical issues, aiming to reduce nations' dependence on fossil fuels. Biofuel production holds significant economic, social (food security and local empowerment), and environmental risks (such as loss of biodiversity and water shortage). Research on the use of vegetable oils as a diesel fuel has been intense during periods of petroleum shortages such as World Wars I and II and the energy crisis of the 1970s [1]. Many countries are using edible oil-seed crops such as soybean, groundnut, sunflower, and oil palm for producing biodiesel.

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Table 49.1 Characteristics of the main oil crops for biodiesel production (*adapted from Ref. [3]*, reprinted with kind permission from ASA, CSSA, and SSSA Book Publishing).

Plant species	Source of oil	Oil content (%)
Aconitum flavum	Seed	46.9
Arachis hypogaea (peanut)	Kernel	40–43
Attalea speciosa	Seed	66.0
A. indica (neem)	Kernel	25–45
Brassica spp. (canola)	Seed	40–48
Calophyllum inophyllum	Kernel	75
Carthamus tinctorius (safflower)	Seed	32–40
Cocos nucifera (coconut)	Fruit	55–60
Elaeis guineensis	Kernel	45–55
Garcinia indica	Kernel	41
Glycine max (soybean)	Seed	20
Helianthus annus (Sunflower)	Seed	38–48
Hevea brasiliensis	Seed	40
J. curcas	Seed	25–40
Madhuca pasquieri	Kernel	46.6
M. phillippinensis	Seed	60
Mesua ferra	Seed	70
Persea americana (avocado)	Fruit	7–35
P. pinanta	Seed	25–40
R. communis (castor)	Seed	45-48
S. album (sandalwood)	Kernel	62.6
Salvadora	Seed	40
Sesamum indicum	Seed	51
Shorea robusta	Kernel	18
Simarouba glauca	seed	65
Simmondsia chinensis (Jojoba)	seed	54
Terminalia catappa	Kernel	59
Thevetia peruviana	Kernel	72.4
Vernicia montana	Kernel	52.5

Table 49.1 summarizes woody oil plant resources for biodiesel production. The European Union is the global leader in biodiesel production and use, with Germany and France accounting for 88% of world production, followed by the United States, which produces 8% of global production [2, 3]. As a feedstock, about 84% of the world's biodiesel production comes from rapeseed oil. However, a prolonged use of such edible oil in many developing countries will lead to shortage of food, especially to the poor, by adversely affecting food supplies and prices. Moreover, agricultural land or lands that are valuable for biodiversity may also be used for cultivating biofuel crops creating a severe competition with food crops [3].

In this context, primary thrust is on exploitation of underutilized nonedible oil crops as a feedstock for biodiesel production. There are many such nonedible oleaginous plants such as neem (*Azadirachta indica* A.), karanja (*Pongamia pinnata*

L.), mahua (*Madhuca* spp.), castor bean (*Ricinus communis* L.), simarouba (*Simarouba glauca* DC.), physic nut (*Jatropha curcas* L.), and so on [4]. Among these crops, *J. curcas* has gained importance since it is a nonfood plant, it does not put any pressure on food supply, and it can be cultivated on wasteland instead of agricultural land.

J. curcas, a perennial shrub, belonging to the Euphorbiaceae family is one of the 170 known species of the genus *Jatropha*. *J. curcas* thought to have been native of South America is widely distributed in the wild or semicultivated areas in Africa, Asia, and parts of Europe. *J. curcas* is a shrub that can reach a height of 8–10 m. The plant root system proceeds through the development of a main taproot and four shallow lateral roots. It typically grows between a temperature range of 15–40 °C with a rainfall between 250 and 3000 mm. However, it is an open field plant species that requires intense sunshine. *J. curcas* is well adapted to arid and semiarid climates with a demonstrated ability to drought tolerance. It can also grow on a wide range of soils provided they are well drained and aerated. Seeds contain 30–35% nonedible oil that can be easily converted to biodiesel that meets the American and European standards. The profile of fatty acid composition is C14:0 (1.4%), C16:0 (15.6%), C18:0 (9.7%), C18:1 (40.8%), C18:2 (32.1%), and C20:0 (0.4%). The seeds can also be used for manufacturing other useful products such as candles, high-quality soaps, and cosmetics as well as for healing several skin disorders. Because of its several industrial and medicinal uses, initial investments toward commercial-scale plantations of this plant are under process. As per the Indian planting system, tree density for commercial cultivation is 2500 ha^{-1}. The total seed production ranges from 2000 to 4000 kg ha^{-1} depending on the genotype.

49.2
Industry Participation in Basic Research and Cultivation of *Jatropha*

Considering its importance, several companies from many countries all over the world have been involved in the development and propagation of *J. curcas* large-scale plantation ventures and basic research. In India, DaimlerChrysler AG has been involved in *Jatropha* research since 2003 in collaboration with an Indian research institution on *J. curcas* cultivation along 2500 km of Indian railroads. Experiments have been conducted along with Central Salt and Marine Chemicals Research Institute (CSMCRI) located in Gujarat in India and University of Hohenheim, Germany. Many companies such as Australia's Mission New Energy Ltd. have invested in India and have taken the initiative for feedstock procurement. The Chinese government is also playing an active role in the development of these biofuels. Chinese oil companies such as China National Petroleum Corporation (CNPC), China Petroleum and Chemical Corporation (SINOPEC), and China National Offshore Oil Corporation (CNOOC) are involved in large-scale investments for plantations of *J. curcas* in provinces of Sichuan, Yunnan, Guizhou, Guangxi, Guangdong, and Hainan. Major commercial airlines are also joining in to boost the biofuel market. Air New Zealand, British Airways, Virgin Atlantic Airways, Japan

Airlines, Boeing Company, and Pratt &Whitney are some of the companies that are planning and conducting test flights using biofuels made of *J. curcas*. There are also a number of *J. curcas* plantation development programs being run by Japanese companies such as Nippon Biodiesel Fuel Co. Ltd., IS Corporation Ltd., and Japanese Bioenergy Development Corporation [5]. Toyota Tsusho Corporation, the parent company of Toyota Motor Corporation, is investing in the cultivation of *Jatropha*, which Toyota intends to refine into biodiesel. Carbon Credited Farming (CCF) joined hands with Crest Global Green Energy to develop *Jatropha* plantations in Mali and Senegal. NASA recently experimented with accelerating the production and breeding process of *Jatropha* by sending *Jatropha* cells and cultures to space in Endeavor Space Shuttle. NASA's decision to experiment with accelerating the production process of *Jatropha*-based biofuels underscores the validity and urgent need for the production and mass implementation of biofuels in the marketplace.

In India, Indian Oil Corporation and Ruchi Soya Industries have forged a partnership to establish 50 000 ha of *Jatropha* plantations and a 31.5 Mgy (119 million L) biodiesel plant. General Motors and the US Department of Energy (DOE) have formalized a 5 year partnership with India's CSMCRI to develop *Jatropha* as a sustainable biofuel energy crop. The goal of the project is to explore the development of new varieties of the plant that have high yields and can withstand adverse environmental conditions.

Tata Chemicals Ltd. partnered with JOil, a Singapore-based *Jatropha* seedling company, to invest US$ 25 million for the production of quality seedlings for its own cultivation in India and East Africa. JOil has been set up by the Temasek Life Sciences Laboratory in Singapore. The other companies involved in *J. curcas* cultivation are Viridas plc. (www.viridasplc.com), Energem Resources Inc. (www.energem.com), and Genetwister (http://www.Genetwister.com).

S.G. Biofuels, a California-based company, has invested in *J. curcas* plantations in Latin and Central America. In 2010, they launched JMax 100 cultivar optimized for growing conditions in Guatemala with yields 100% greater than existing varieties. S.G. Biofuels is applying 1.6 million SNP markers for *J. curcas* toward the development of marker-assisted selection (MAS) methods to produce elite, high-performing cultivars. The company claims to have more than 12 000 unique genotypes of *Jatropha*, the largest and most diverse library of *Jatropha* genotypes in the world.

In Indonesia, the cultivation of *J. curcas* as a biodiesel crop was started by a company called P.T. Agrila in 2003 on the island of Lombok. It experienced a boom in cultivation when the crude oil prices went up in 2006. In 2006, the Indonesian government announced the "Blueprint: Biofuel development" as a measure against poverty and unemployment. Under the program, it was planned that 500 000 people would be employed and 7.5 million ton of *J. curcas* oil would be produced in 2010 and 1 million people would be employed and 15 000 000 ton of crude *Jatropha* oil would be produced by 2015. Myanmar is also emphasizing the development of *J. curcas* as a biodiesel crop and had planned to cultivate 2.3 million ha by 2010 [5].

The present interest by various public and private sector companies highlights the importance of *Jatropha* biodiesel as a source of alternative energy source.

49.3
Projections of *Jatropha* Cultivation

The International Jatropha Organization has announced cultivation plans and projections for different countries in each part of the world over the next 10 years. It claims that by 2017 there will be 32.72 million ha of land cultivated with *J. curcas* worldwide producing 160 million ton of seeds. According to this organization's projections for Asia, the cultivation plans of China, India, Indonesia, and Myanmar are the most prominent [5]. The cultivation in Asia is the highest at 31.2 million ha, followed by Africa at 1.36 million ha, Middle East at 0.1 million ha, and Latin and Oceania with 1000 ha each.

49.4
Challenges for Commercial-Level Success

According to an analysis by Weyerhaeuser *et al.* [6] the challenges for developing viable markets for *Jatropha* oil are considerable. The highlights are as follows:

- Ensuring sufficient quantity and quality of available, nonagricultural and non-forest land to meet a reasonable scale of feedstock demand;
- Building institutions that facilitate between smallholder farmers upstream and the oil and biodiesel processing industries downstream;
- Determining minimum efficient scales for *Jatropha* growing and processing;
- Coordinating the timing of government investments in *Jatropha* research with the speed of the *Jatropha* biodiesel industry's development, and ensuring that the scale of implementation matches the appropriate scale suggested by research results.

49.5
Target Traits for *J. Curcas* Crop Improvement

J. curcas chromosomes are very small in size with most species having 22 chromosomes. It has been an attractive crop for whole-genome sequencing due to its modest genome size of 416 Mbp [7]. Synthetic Genomics Inc. (SGI) and Asiatic Center for Genome Technology (ACGT) have announced the completion of the first draft of sequencing project. Sequencing revealed that *J. curcas* has 400 million bp, which is similar to rice. Almost at the same time, Sato *et al.* [8] reported whole-genome sequencing by a combination of Sanger method and 454 next-generation sequencing. Sequencing the genome of *J. curcas* will be useful in identifying genes of interest and provide information on factors controlling oil synthesis, maximizing yield, biotic and abiotic stress tolerance, and different toxins [8].

Genetic transformation studies using *Agrobacterium* [9, 10] and particle bombardment [11] have been reported. A number of traits have been identified for *J. curcas*

crop improvement; the traits such as seed yield, oil content, seed toxicity, female to male flower ratio, increase in branching, early flowering, synchronous maturity, and adaptation to abiotic stresses are considered relevant for the success of the commercial-scale plantations [12]. Improvement in seed yield could be achieved by increasing the ratio of female flowers [12]. Increased seed yield was achieved by increasing the ratio of lateral branches [13] and by application of paclobutrazol, a growth regulator [14]. Yield loss due to pests such as *Scutellera nobilis* and *Pempelia morosalis* has also been observed in some *Jatropha* monocultures. Pest- and pathogen-resistant varieties can considerably increase yields.

49.6
Fatty Acid Biosynthesis and Abiotic Stress Tolerance in *J. Curcas*

49.6.1
Fatty Acid Biosynthesis Genes

Identification of cDNAs involved in fatty acid biosynthesis and biotic/abiotic stresses through EST profiling is becoming popular and has gained importance. Costa *et al.* [15] generated 13 249 ESTs from developing and germinating *J. curcas* seeds. This enabled them to detect major key genes involved in lipid synthesis and degradation. Among the most abundantly expressed transcripts they found transcripts related to breakdown of the oil and carbohydrate reserves such as acetyl-CoA C-acyltransferase. Most ESTs coding for fatty acid biosynthesis enzymes, for example, *FatA*, *FatB*, *ACC*, *FAD2*, *KAS I*, *KAS II*, and so on (acyl-ACP thioesterase A, acyl-ACP thioesterase B, acetyl-CoA carboxylase, oleoyl-ACP desaturase, ketoacyl-ACP synthase I, and ketoacyl-ACP synthase II, respectively) were found in developing seeds and for fatty acid degradation pathway, *FADA*, *ATOB*, *ADH*, *ACADM*, and so on (aceyl-CoA acyltransferase, acetyl-CoA C-acetyl transferase, alcohol dehydrogenase, and acyl-CoA dehrdogenase, respectively) were found in germinating seeds of *J. curcas*. The most important enzyme was found to be oleoyl-ACP desaturase (*FAD*) that catalyzes the polyunsaturation of oleoyl-ACP (18: 1) to linoleoyl-ACP (18: 2). Since oleic and linoleic acids are the major constituents of *J. curcas* oil, this enzyme can be used for modulation of oil composition [15].

On similar lines, Gomes *et al.* [16] studied the expression levels of four genes (palmitoyl-acyl carrier protein thioesterase, 3-ketoacyl-CoA thiolase B, lysophosphatidic acid acyl transferase, and geranyl pyrophosphate synthase) involved in pathways of biosynthesis of fatty acids, biosynthesis of unsaturated fatty acid, glycerolipid metabolism, and biosynthesis of terpenoid backbones, respectively. The genes were more expressed in fruits than in leaves. This information can be useful for selective breeding for traits in relation to biodiesel production and low-phorbol varieties [16]. Knowledge of expression overview of multiple genes involved in oil biosynthesis from different stages and tissues of *J. curcas* is the approach adopted by Xu *et al.* [17]. In their study, they analyzed the expression profiles of 21 genes involved in different steps of the pathway leading to fatty acid and TAG synthesis at different time points of

seed development and compared it with leaf. They found the expression levels of 17 genes significantly higher in seeds compared to leaves. In a very recent study, Popluechai *et al.* [18] studied the proteome composition of oil bodies in the seed of *J. curcas*. The study revealed oleosins as the major component and additional proteins similar to those in other oil seed.

Several reports have been published to identify genes involved in the fatty acid biosynthesis pathway and that can be used to produce transgenic *Jatropha* plants to obtain new improved varieties with increased oil content. Increasing the oil content in seeds can be achieved by altering the expression levels of enzymes in the triacylglycerol (TAG) biosynthetic (Kennedy) pathway. Overexpression of diacylglycerol acyl transferases has been shown to increase oil content in *Arabidopsis* [19] and soybean [20]. The regulation of seed development and TAG biosynthesis in seeds has been studied in some depth [21]. Stearoyl-acyl carrier protein desaturase is an important enzyme for fatty acid biosynthesis in higher plants, which determines the ratio of saturated to unsaturated fatty acids. Tong *et al.* cloned the above gene from developing seeds of *J. curcas* and functionally expressed it in *Eshcerichia coli* [22]. Furthermore, β-ketoacyl-acyl carrier protein (ACP) synthase III (*KAS III*) is believed to be involved in carbon chain elongation. Li *et al.* cloned *KAS III* from *J. curcas*, which they termed *JcKASIII* [23]. The authors concluded that the study might help in understanding fatty acid biosynthesis in *J. curcas*. Likewise, gene involved in termination of carbon chain elongation was studied. Wu *et al.* cloned *JcFATB1* from *J. curcas*, which encodes a putative acyl-ACP TE (involved in termination of carbon chain). When *JcFATB1* was cloned in *Arabidopsis thaliana*, the saturated fatty acid content increased [24]. These results indicate that *JcFATB1* might have a role in altering the fatty acid composition.

Lipid profiling of developing seeds of *J. curcas* was carried out using ^{1}H NMR spectroscopy [25]. The oil content in seed samples ranged from 0.3 to 24.9%, lowest being at stage I (7 days after pollination) and highest at stage IV (32 days after pollination). It was concluded that free fatty acids (FFA) contributed predominantly to the total lipids in early stages of development, whereas these were replaced by triglycerol esters at the maturing stages.

Acetyl CoA carboxylase (ACCase) is biotinylated enzyme that catalyzes the ATP-dependent carboxylation of acetyl CoA to form malonyl CoA. This is the first committed step in the biosynthesis of fatty acids. A full-length cDNA of the carboxyl transferase (*accA*) gene of *ACCase* from *J. curcas* was cloned by Xie *et al.* [26]. Fluorogenic real-time polymerase chain reaction (RT-PCR) studies on fruits showed a significant increase in *accA* activities under dark condition compared to the control. The findings suggested that the expression levels of the *accA* gene are closely related to the growth conditions and developmental stages in the leaves and fruits of *J. curcas*.

Seed oil of *J. curcas* is also a source of toxins such as phorbol esters and curcin [27]. Phorbol esters have certain beneficial effects as well. Reports indicated that phorbol esters exhibit insecticidal and molluscicidal activities [27]. Lin *et al.* characterized gene 3-hydroxy-3-methylglutaryl coenzyme A reductase (*JcHMGR*) from *J. curcas*. *JcHMGR* acts as one of the key regulatory enzymes for phorbol esters biosynthesis in the cytosolic mevalonic acid pathway. Therefore, *JcHMGR* may be a target gene

Table 49.2 Partial list of genes cloned and characterized in *J. curcas*. Some of the genes have role in fatty acid synthesis.

No.	Name of gene	Length (bp)	References
1.	Aquaporin	843	[34]
2.	3-Hydroxy-3-methylglutaryl coenzyme A	1950	[27]
3.	Geranylgeranyl diphosphate synthase	1110	[28]
4.	Curcin 2	927	[51]
5.	JcERF	774	[43]
6.	Betaine aldehyde dehydrogenase	1509	[35]
7.	Curcin	882	[36]
8.	β-ketoacyl-acyl carrier protein synthase III	1203	[9]
9.	JcFATB1	1257	[24]
10.	Stearoyl-acyl carrier protein desaturase	1191	[22]
11.	Plastidial ω3 fatty acid desaturase	1368	[46]
12.	Superoxide dismutase		[39, 40, 46]
13.	Peroxidase		[39, 40, 46]
14.	Catalase		[39, 40, 46]
15.	Phenylalaline ammonia-lyase		[39, 40, 46]
16.	Carboxyl transferase or ACCase	1149	[26]
17.	Allene oxide cyclase	924	[48]
18.	Phospholipase D	2427	[49]

in metabolic engineering to alter the content of phorbol estrers [27]. On similar lines, another gene encoding geranylgeranyl diphosphate synthase (*Jc-GGPPS*) was also cloned and characterized [28]. The gene was involved in mediating the biosynthesis of carotenoid and general precursor for diterpenes' biosynthesis. The results provide insight into regulation of phorbol ester biosynthesis in *J. curcas*. A number of studies have indicated that it is possible to increase the oil content of seeds by manipulating of the expression levels of key regulators of seed oil accumulation. For example, disruption of the homeobox gene *GLABRA2* led to increased oil content in *Arabidopsis* [29] and overexpression of soybean transcription factor *GmDOF4* and *GmDOF11* in *Arabidopsis* has also been shown to result in increased oil content [30]. The partial lists of genes cloned that are involved in fatty acid biosynthesis have been summarized in Table 49.2.

49.6.2
Genes Associated with Abiotic Stress Tolerance

J. curcas is capable of growing under arid and semiarid regions on marginal lands. However, under these conditions, it is known to complete its life cycle without commercially acceptable yields. To understand and characterize the genes involved during abiotic stress conditions, Eswaran *et al.* [31] developed functional genetic screen using *shs* (salt hypersensitive) mutant of yeast. Initially, three *shs* mutants have been generated by random mutagenesis that exhibited growth retardation beyond

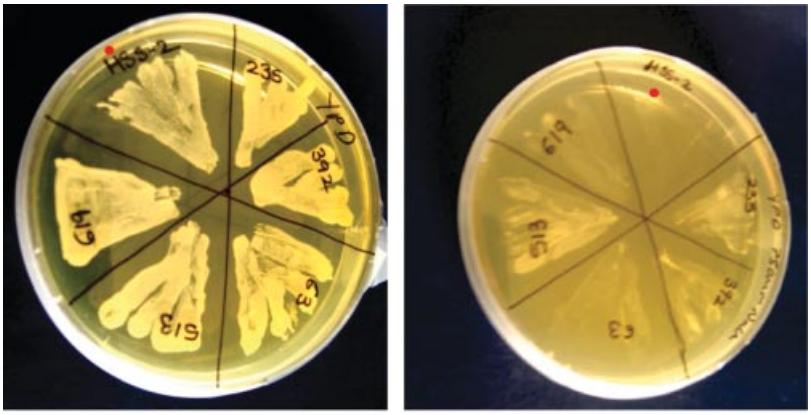

Figure 49.1 Screening and functional evaluation of *J. curcas* genes in salt-hypersensitive (*shs-2*) yeast mutant. *shs-2* mutants were selected by random mutagenesis that has salt sensitivity beyond 500 mM NaCl, whereas plasmids containing stress-tolerant genes can grow under high salt (750 mM NaCl) in synthetic media. (a) Growth of *shs-2* mutant (filled circle) at 0 mM NaCl. (b) Arrest of the *shs-2* mutant growth was seen at high salt level (filled circle), whereas plasmids containing late-embryogenesis abundant protein-5 (235), cytosolic ascorbate peroxidase-1 (392), and metallothionein (513) were found growing under high salt conditions. This screen provides evidence for rapid screening of cDNA library of *J. curcas* for abiotic stress-tolerant genes using yeast mutants (adapted from Ref. [31]).

500 mM NaCl. These mutants have been used to screen cDNA library generated from salt stressed roots of *J. curcas* (Figure 49.1). The authors obtained several full-length functional genes expressed in response to abiotic stress conditions (Table 49.3). Gene expression analysis of five selected genes in leaf and root tissues of *J. curcas* was studied in order to correlate gene expression pattern under imposed stress conditions [31]. The selected genes were late-embryogenesis abundant protein-5 (*LEA-5*), cytosolic ascorbate peroxidase (*Apx-1*), metallothionein, profilin, and annexin. Analysis of the gene expression data suggests dynamic changes in the transcript abundance of these genes, with changes in transcript level being apparent from 2 h time point, indicating an early regulation of the genes in response to salt stress. Gene expression of the normalized transcripts suggests both upregulation and repression of these transcripts. Increased expression of profilin was noted in root tissue between 2 and 8 h time points of exposure to 150 mM salt stress, whereas in leaves profilin transcript was repressed (Figure 49.2a and b), consistent with previously reported function during salt stress [32]. Furthermore, in contrast, an initial repression was observed in the expression of annexin that was subsequently induced in the root tissue, while the expression was downregulated upon salt stress in the leaves (Figure 49.2a and b). Transcript abundance in the levels of *Apx-1* and metallothionein were downregulated in roots, while a more dynamic expression pattern was observed in the leaf tissues, implicating a role in stress-adaptive

Table 49.3 Cloning of abiotic stress-responsive genes derived from salt-stressed root cDNA library of *J. curcas* [31].

No.	Function of the gene	Length of gene (bp)	GenBank Accession No.
1.	Allene oxide cyclase	777	FJ489601
2.	Thioredoxin H-type (*TRX-h*)	357	FJ489602
3.	Metallothionein	234	FJ489603
4.	Heterotrophic ferredoxin	492	FJ489604
5.	Defensin	234	FJ489605
6.	Calmodulin-7 (*CAM-7*)	810	FJ489606
7.	Major allergen Pru ar1-like protein	495	FJ489607
8.	S18.A ribosomal protein	495	FJ489608
9.	60S ribosomal protein L18a	537	FJ489609
10.	Protease inhibitor/seed storage/lipid transfer protein family	348	FJ489610
11.	Membrane protein -2	189	FJ619041
12.	Late-embryogenesis abundant protein 5	267	FJ619042
13.	Cold-induced plasma membrane protein	174	FJ619043
14.	Cytosolic ascorbate peroxidase -1 (*Apx-1*)	753	FJ619044
15.	Profilin-like protein	384	FJ619045
16.	Caffeoyl-CoA-*O*-methyltransferase (*CCoAOMT*)	741	FJ619046
17.	Eukaryotic translation initiation factor SUI1	381	FJ619047
18.	Copper chaperone	282	FJ619048
19.	Ubiquitin conjugating enzyme 2 (*JcE2*)	447	FJ619049
20.	Mitochondrial ATP synthase 6 KD subunit (*JcMtATP6*)	171	FJ619050
21.	Ferritin-2, chloroplast precursor	771	FJ619051
22.	Annexin-like protein	945	FJ619052
23.	Al-induced protein	711	FJ619053
24.	Avr9/cf-9 rapidly elicited (*JcACRE*) gene	231	FJ619054
25.	40S ribosomal protein S15	456	FJ623457
26.	Plant lipid transfer/seed storage/trypsin-alpha amylase inhibitor	306	FJ623459
27.	Low molecular weight cysteine-rich 69	234	FJ623460

responses. Among the transcripts profiled in this study, only *LEA-5* was upregulated in leaves at the 8 h time interval, after initial downregulation at the 2 h time point, while in roots *LEA-5* expression was unaffected under salt stress. Such an induction of *LEA-5* in leaf tissue demonstrated either a longer-range signal or the terminal effects of long exposure to ions taken by the roots. Further experiments are required to delineate these effects. The limited gene expression data, indirectly suggests varied modes of gene regulation between the root and the leaf tissue in *J. curcas* (Figure 49.2a and b). The data indicates a complex framework for gene regulation during adaptation to salt stress in different tissues.

Aquaporins are membrane proteins or major intrinsic proteins (MIPs) that form water channels or pores in biological cell membranes, controlling the water content of the cells. These channels are widely distributed in all kingdoms of life including

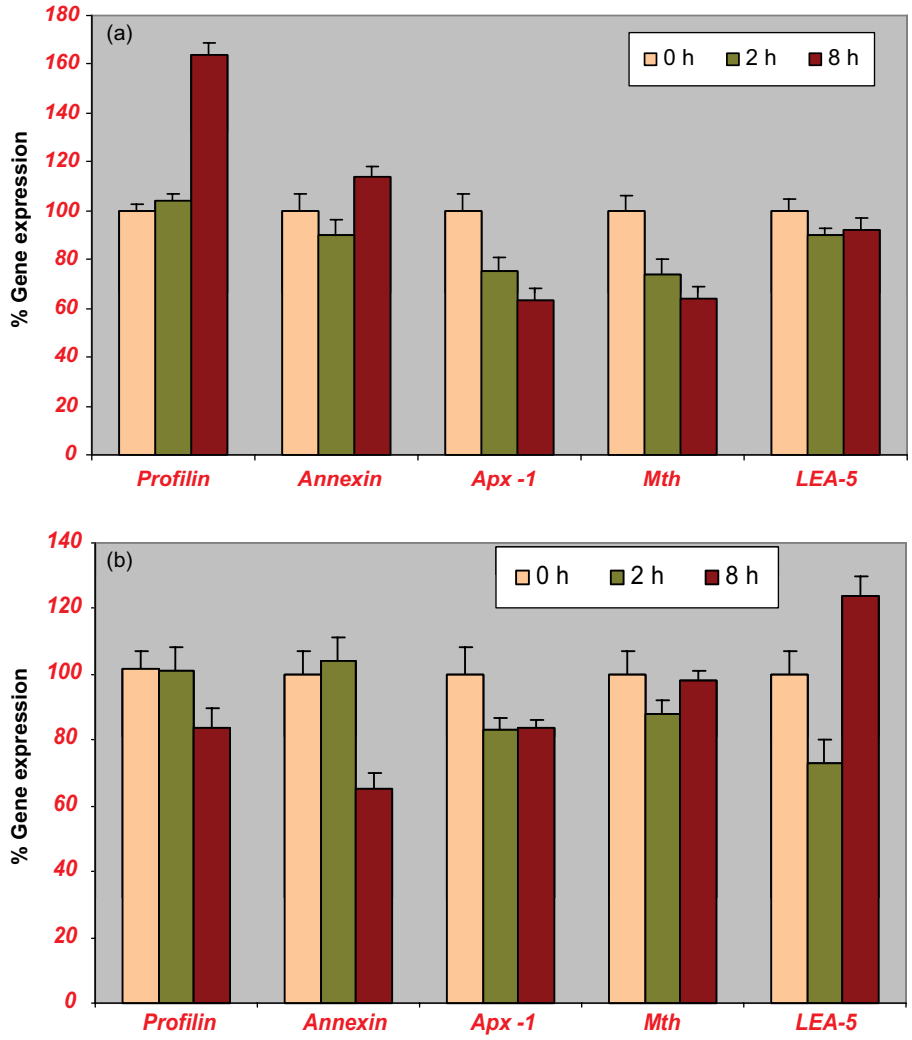

Figure 49.2 (a) Semiquantitative RT-PCR expression analysis of five different genes of *J. curcas* in root tissue after treating *J. curcas* seedlings with 150 mM NaCl. Semiquantitative RT-PCR was performed at 0 h, 2 h, and 8 h of exposure to NaCl. *Apx-1*, cytosolic ascorbate peroxidase-1; *Mth*, metallothionein; *LEA-5*, late-embryogenesis abundant protein-5. Bars represent SE of mean (adapted from Ref. [31]). (b) Semiquantitative RT-PCR expression analysis of five different genes of *J. curcas* in leaf tissue after treating *J. curcas* seedlings with 150 mM NaCl. Semiquantitative RT-PCR was performed at 0 h, 2 h, and 8 h of exposure to NaCl. *Apx-1*, cytosolic ascorbate peroxidase-1; *Mth*, metallothionein; *LEA-5*, late-embryogenesis abundant protein-5. Bars represent SE of mean (adapted from Ref. [31]).

bacteria, plants, and mammals [33]. In plants, aquaporins are present in multiple isoforms. Aquaporins are thought to be involved in plant adaptation to drought stress, controlling transmembrane water movement in plants. In order to study the role of aquaporin in drought stress, a full-length cDNA encoding aquaporin (*JcPIP2*) was isolated from seedlings of *J. curcas*. Heavy drought conditions were induced by PEG6000 to study its role. It was observed that with increasing levels of drought stress *JcPIP2* level increased indicating its role in drought resistance [34]. The presence of aquaporins is considered to play an important role in the rapid growth of *J. curcas* under dry weather conditions. In another study, betaine aldehyde dehydrogenase (BADH) gene called *JcBD1* (cloned by RT-PCR and RACE techniques) has been isolated from *J. curcas* [35]. It was observed that *JcBD1* gene transcript levels were 79% higher in case of plants exposed to drought, salt, and heat stress compared to control plants, indicating its role in all three abiotic stress tolerance. Functional expression of *JcBD1* in *E. coli* conferred resistance to salt stress [35].

J. curcas is known to be a toxic plant. Curcin, a ribosome inactivating protein (RIP), was found in the seeds of this plant. The full-length *curcin* gene has been cloned and sequenced [36]. In one study, curcin-L, a type I RIP, was isolated from *J. curcas*. Its expression could be induced in leaves by administering treatments with abscisic acid, salicylic acid, PEG, temperature (45 °C), and ultraviolet radiation. The gene transcripts were not detected in stem, roots, and seeds. A 654 bp fragment of a 5′ flanking region preceding the *curcin-L* gene, designated *CP2*, was cloned from *J. curcas* and its expression was studied in transgenic tobacco via the expression of β-glucuronidase (*GUS*) gene. It was found that CP2 was leaf specific and could induce the expression of the reporter gene under stress conditions [37].

In another study recently conducted on seedlings of *J. curcas* treated with increasing concentrations of NaCl, it was found that superoxide dismutase (SOD) activity in the cotyledons, hypocotyls, and radicles increased gradually up to NaCl concentrations of 150, 200, and 150 mmol by 70.8%, 86.8%, and 72.8%, respectively, compared to controls [38]. SOD is one of the several important antioxidant enzymes with the ability to repair oxidative damage caused by reactive oxyzen species (ROS). Similarly, peroxidase (POD) activity in the cotyledons, hypocotyls, and radicles was highest at NaCl concentrations of 150, 200, and 150 mmol by 52, 122.2, and 62.2%, respectively, compared to controls. POD is involved in various processes including lignification, auxin metabolism, salt tolerance, and heavy metal stress. Catalase (CAT) activity in cotyledons, hypocotyls, and radicles increased gradually up to NaCl concentrations of 100, 200, and 150 mmol by 75.4, 246.9, and 588.1%, respectively, compared to controls. CAT, which is involved in the degradation of hydrogen peroxide into water and oxyzen, is the most effective antioxidant enzyme against oxidative damage. Therefore, increased activity of CAT along with SOD and POD plays an important role in ROS scavenging process and salt-induced oxidative stress tolerance. Another enzyme phenyalanine ammonia-lyase (PAL), a marker in environmental stress also showed increased activity in cotyledons, hypocotyls, and radicles at NaCl concentrations of 150, 200, and 150 mmol, respectively, compared to controls [38].

Effect of high concentrations of metals such as nickel, zinc, and lead was studied. Reports indicate high concentration of nickel (100, 200, 400, and 800 μmol) (heavy

metal stress) on *J. curcas* seedlings is correlated with the activity levels of SOD, POD, CAT, and PAL antioxidant enzymes. It was found that the activity of SOD, POD, and CAT had a negative correlation with nickel concentrations with the activity highest at 400, 200, and 200 μmol of nickel. PAL had a positive correlation with the highest activity at 400 μmol of nickel. Hence, the lower nickel concentrations and higher SOD, POD, CAT, and PAL activities suggest the tolerance capacity to protect the plant from oxidative damage due to heavy metal stress [39]. Effect of different zinc concentrations on *J. curcas* seedlings and its influence on antioxidant enzymes such as SOD, POD, CAT, and PAL have also been reported [40]. Authors found that the biomass of cotyledons, hypocotyls, and radicles was increased with increasing concentrations of zinc. SOD and POD activity was increased gradually with the increase in zinc concentrations. CAT activity in cotyledons, hypocotyls, and radicles reached the largest increments at the zinc concentrations of 0.5, 2, and 0.5 mM, respectively. A similar trend was observed in case of PAL activity when compared to CAT. Hence, it is seen that SOD, POD, CAT, and PAL may also play an important role in the defense mechanisms of *J. curcas* exposed to excess metal [40]. In another similar finding, it was reported that increased SOD, POD, and PAL activity is involved in the defense mechanism of *J. curcas* radicles against lead toxicity [41]. Changes in protein content, SOD, CAT, POD, and PAL in *J. curcas* seedlings showed a clear correlation with copper concentrations. The results of this study also show that the ability of *J. curcas* to cope with metal stress depends on oxidative stress defense mechanisms [42].

Transcription factors and regulatory elements have a major role in manipulation of the expression of key genes during abiotic stress tolerance and has been a major focus of study. Tang *et al.* [43] characterized *JcERF* gene, a putative AP2/EREBP domain containing transcription factor, from *J. curcas*. The AP2/EREBP proteins in plants are the largest transcription factor family having a role in plant development and responses to ethylene, disease, and other biotic and abiotic stresses. In *J. curcas*, it was noticed that expression of the *JcERF* gene was rapidly induced upon salinity, drought, ethylene, and mechanical wounding proving that in *J. curcas* the gene has a role in tolerance to abiotic stresses. Photosynthetic characteristics and chlorophyll fluorescence parameters have also been studied in *J. curcas* under drought stress [44] and cold stress [45]. Under drought stress, it was observed that when treated with lower concentrations ($\leq$15%) of PEG, the photosynthetic rate (P_n), stomatal conductance (G_s), and intercellular carbon dioxide concentration (C_i) of the seedlings decreased with increasing PEG concentration, while the chlorophyll fluorescence parameters did not change. There was a drop in PSII activity. When drought stress was removed, there was recovery of PSII activity. The results demonstrated that *J. curcas* has strong tolerance to drought stress [44]. Similarly, under cold stress (4 °C) eight photosynthesis-related proteins significantly changed. The chlorophyll fluorescence parameters were also sensitive to cold stress. There was a correlation between photosynthesis-related proteins and chlorophyll fluorescence parameters indicating that the early-stage (0–12 h) acclimation of PSII and the late-stage (after 24 h) H_2O_2 scavenging might be involved in cold response mechanisms in *J. curcas* [45].

Guo *et al.* [46] cloned a plastidal ω3 fatty acid desaturase gene from leaves of *J. curcas*, which is involved in the synthesis of trienoic fatty acids. It is known that high ω3 fatty acid content in leaves can increase the plant's tolerance to cold stress. Hence, by identifying this gene in *J. curcas* it will be useful to understand the molecular mechanism of cold tolerance in *J. curcas* and will help in future to improve its tolerance to cold stress. It is known that soil alkalinity can also be a stress to plants. When the effect of alkalinity (Na_2CO_3 0.1, 0.2, 0.3, 0.4, and 0.5%) was studied on *J. curcas* seedlings, it was observed that increase in alkalinity caused reduction in growth. But when some beneficial microbes (*Azotobacter microfoss* and arbuscular mycorrhizal fungi (AMF)) were added in combinations, it was observed that different combinations of these microbes with 0.4% sodium carbonate increased the survival percentages compared to control plants. The combination of AM fungi and *Azotobacter* increased plant height, shoot diameter, shoot dry weight, leaf relative water content, and soluble sugar content and decreased level of soluble protein at 0.4% of Na_2CO_3 compared to other treatments [47].

Allene oxide cyclase (AOC) is a key enzyme in jasmonates biosynthesis pathway. A cDNA encoding AOC, from *J. curcas* named *JcAOC* has been cloned [48]. Semiquantitative RT-PCR analysis revealed that *JcAOC* mRNA was expressed in roots, stems, leaves, young seeds, endosperms, and flowers, but that the expression level was highest in leaves and lowest in seeds, and mRNA expression of *JcAOC* could be induced by salt stress (300 mM NaCl) and low temperature (4 °C) [48]. Phospholipase D (PLD) is a key enzyme in plants involved in phospholipid catabolism, initiating a lipolytic cascade in membrane deterioration during senescence and stress. Liu *et al.* [49] cloned 2886 bp full-length phospolipase D cDNA from *J. curcas*. Phylogenetic analysis indicated that the *J. curcas* PLD alpha (*JcPLDα*) showed a high similarity to PLD alpha from other plants. Semiquantitative RT-PCR analysis revealed that it was especially abundant in root, stem, leaf, endosperm, and flower and weakly in seed. And the *JcPLDα* was increasingly expressed in leaf undergoing environmental stress such as salt (300 mM NaCl), drought (30% PEG), cold (4 °C), and heat (50 °C). The *JcPLDα* protein was successfully expressed in *E. coli* and showed high enzymatic activities.

49.7 Future Perspectives and Conclusions

Nonedible oil crops, especially, *J. curcas* hold promise as a substitute for fossil fuel and has a prominent future. However, large-scale production without the application of improved farming techniques and elite breeds has diverse problems. With the availability of genome sequence, molecular breeding programs can be accelerated to improve yields.

A recent study to understand oil mobilization in germinating seeds, proteomic analysis of endosperm clearly showed that oil mobilization that was initiated during germination was used up for early seedling development [50]. Several pathways including β-oxidation, glyoxylate cycle, glycolysis, citric acid cycle, gluconeogenesis,

and pentose phosphate pathway were involved in the oil mobilization. These results would benefit to further understand the mobilization and control of oil in the seed.

The future of *J. curcas* as a sustainable energy crop will largely depend on successful integration of "omics" approach with metabolic engineering. The efforts at the authors' lab as detailed in Table 49.3 in identifying abiotic stress tolerance-governing factors have led to major discovery of transcriptional elements and genes in the stress tolerance pathway. The genes and outcome of the results can be successfully utilized to molecular breeding of stress-resistant *J. curcas* in particular and other biofuel crops in general.

References

1 Duffield, J., Shapouri, H., Graboski, M. *et al.* (1998) US Biodiesel Development: New Markets for Conventional and Genetically Modified Agricultural Fats and Oils. Agricultural Economic Report No. 770 USDA, Economic Research Service, Washington DC.

2 Hazell, P. and Pachauri, R.K. (2006) Overview, in *Bioenergy and Agriculture: Promises and Challenges, Focus 14* (eds P. Hazell and R.K. Pachauri), Int. Food Policy Res. Instt., Washington DC.

3 Nass, L.L., Pereira, P.A.A., and Ellis, D. (2007) *Crop Sci.*, **47**, 2228–2237.

4 Carels, N. (2009) *Adv. Botanical Res.*, **50**, 39–86.

5 Siang, C.C. (2009) *Jatropha curcas* L.: Development of a new oil crop for biofuels (Summary). The Institute of Energy Economics (IEEJ), Japan. February.

6 Weyerhaeuser, H., Tennigkeit, T., Yufang, S. *et al.* (2007) Biofuels in China: an analysis of the opportunities and challenges of *Jatropha curcas* in Southwest China, ICRAF China 2007, ICRAF Working Paper Number 53.

7 Carvalho, C.R., Clarindo, W.R., Pracxa, M.M. *et al.* (2008) *Plant Sci.*, **174**, 613–617.

8 Sato, S., Hirakawa, H., Isobe, S. *et al.* (2010) *DNA Res.* doi 10.1093/dnares/dsq030

9 Li, M., Li, H., Jiang, H. *et al.* (2008) *Plant Cell Tissue Organs Cult.*, **92**, 173–181.

10 Mazumdar, P., Basu, A., Paul, A. *et al.* (2010) *South Afr. J. Bot.*, **76**, 337–344.

11 Purkayastha, J., Sugla, T., Paul, A. *et al.* (2010) *Biol. Plant.*, **54**, 13–20.

12 King, A.J., He, W., Cuevas, J.A. *et al.* (2009) *J. Exp. Bot.*, **60**, 2897–2905.

13 Abdelgadir, H.A., Johnson, S.D., and Staden, J.V. (2009) *Plant Growth Regul.*, **58**, 287–295.

14 Ghosh, A., Chikara, J., Chaudhary, D.R. *et al.* (2010) *J. Plant Growth Regul.*, **29**, 307–315.

15 Costa, G.G.L., Cardoso, K.C., Bem, L.E.V.D. *et al.* (2010) *BMC Genomics*, **11**, 462.

16 Gomes, K.A., Almeida, T.C., Gesteira, A.S. *et al.* (2010) *Genome Insights*, **3**, 29–56.

17 Xu, R., Wang, R., and Liu, A. (2011) *Biomass Bioenergy.* doi: 10.1016/j.biombioe.2011.01.001

18 Popluechai, S., Froissard, M., Jolivet, P. *et al.* (2011) *Plant Physiol. Biochem.*, **49**, 352–356.

19 Jako, C., Kumar, A., Wei, Y. *et al.* (2001) *Plant Physiol.*, **126**, 861–874.

20 Lardizabal, K., Effertz, R., Levering, C. *et al.* (2008) *Plant Physiol.*, **148**, 89–96.

21 Santos-Mendoza, M., Dubreucq, B., Baud, S. *et al.* (2008) *Plant J.*, **54**, 608–620.

22 Tong, L., Peng, S.M., Deng, W.Y. *et al.* (2006) *Biotechnol. Lett.*, **28**, 657–662.

23 Li, J., Li, M.-R., Wu, P.-Z. *et al.* (2008) *Tree Physiol.*, **28**, 921–927.

24 Wu, P.-Z., Li, J., Wei, Q. *et al.* (2009) *Tree Physiol.*, **29**, 1299–1305.

25 Annarao, S., Sidhu, O.P., Roy, R. *et al.* (2008) *Biosource Technol.*, **99**, 9032–9035.

26 Xie, W.-W., Gao, S., Wang, S-h. *et al.* (2010) *Z. Naturforsch.*, **65c**, 103–108.

27 Lin, J., Jin, Y., Zhou, M. *et al.* (2009) *African J. Biotechnol.*, **8**, 3455–3462.

28 Lin, J., Jin, Y., Zhou, X. *et al.* (2010) *African J. Biotechnol.*, **9**, 3342–3351.

29 Shen, B., Sinkevicius, K., Selinger, D. *et al.* (2006) *Plant Mol. Biol.*, **60**, 377–387.

30 Wang, H.-W., Zhang, B., Hao, Y.-J. *et al.* (2007) *Plant J.*, **52**, 716–729.

31 Eswaran, N., Parmeshwaran, S., Sathram, B. *et al.* (2010) *BMC Biotechnol.*, **10**, 23.

32 Ramachandran, S., Christensen, H.E.M., Ishimaru, Y. *et al.* (2000) *Plant Physiol.*, **124**, 1637–1647.

33 Amiry-Moghaddam, M., Lindland, H., Zelenin, S. *et al.* (2005) *Fed. Am. Soc. Exp. Biol.*, **19**, 1459–1467.

34 Zhang, Y., Wang, Y., Jiang, L. *et al.* (2007) *Acta Biochim. Biophys. Sin.*, **39**, 787–794.

35 Zhang, F.-L., Niu, B., Wang, Y.-C. *et al.* (2008) *Plant Sci.*, **174**, 510–518.

36 Lin, J., Chen, Y., Xu, Y. *et al.* (2003) *Acta Bot. Sin.*, **45**, 858–863.

37 Qin, X., Zheng, X., Shao, C. *et al.* (2009) *Planta*, **230**, 387–395.

38 Gao, S., Ouyang, C., Wang, S. *et al.* (2008a) *Plant Soil Environ.*, **54**, 374–381.

39 Yan, R., Gao, S., Yang, W. *et al.* (2008b) *Plant Soil Environ.*, **54**, 294–300.

40 Luo, Z.-B., He, X.-J., Chen, L. *et al.* (2010) *Int. J. Agric. Biol.*, **12**, 119–124.

41 Gao, S., Li, Q., Ou-Yang, C. *et al.* (2009) *Fresenius Environ. Bull.*, **5**, 811–815.

42 Gao, S., Yan, R., Cao, M. *et al.* (2008b) *Plant Soil Environ.*, **54**, 117–122.

43 Tang, M., Sun, J., Liu, Y. *et al.* (2007) *Plant Mol. Biol.*, **63**, 419–428.

44 Dou, X.-Y., Wu, G.-J., Huang, H.-Y. *et al.* (2008) *Ying Yong Sheng Tai Xue Bao.*, **19**, 1425–1430. Article in Chinese, only abstract was consulted.

45 Liang, Y., Chen, H., Tang, M.-J. *et al.* (2007) *Physiol. Plant.*, **131**, 508–517.

46 Guo, L., Qing, R., He, W. *et al.* (2008) *Chin. J. Appl. Environ. Biol.*, **14**, 469–474.

47 Kumar, A., Sharma, S., and Mishra, S. (2009) *J. Phytology*, **1**, 177–184.

48 Liu, B., Wang, W., Gao, J. *et al.* (2010a) *Acta Physiol. Planta.*, **32**, 531–539.

49 Liu, B., Yao, L., Wang, W. *et al.* (2010b) *Mol. Biol. Rep.*, **37**, 939–946.

50 Yang, M.F., Liu, Y.-L., Liu, Y. *et al.* (2009) *J. Proteome Res.*, **8**, 1441–1451.

51 Qin, W., Huang, M-X., Xu, Y., Zhang, X-S., and Chen F. (2005) *J. Biosci.*, **30**, 351–357.

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Brassica Crop Species: Improving Water Use Efficiency: Challenges and Opportunities

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The *Brassica* species occupies a large portion of the world's economically important cultivated crops. These include vegetables, oilseeds, condiments, and forages. These crops are grown globally under a wide range of climatic conditions. With current concerns for food and energy security, expanded use of crop products, and environmental stewardship, there is a pressing need to improve yield through greater efficiency of resource utilization. Water availability is the most limiting factor to crop productivity and with the predicted scarcity, due to climate change and increased nonagricultural demand, improving water use efficiency (WUE) in crop production is an imperative. Consequently, increased carbon assimilation per unit of water used by *Brassica* crops must not only be realized but this carbon must also be efficiently partitioned into the harvested product. Thus, these plants need to be equipped with the genetic capacity to extract more water from the soil under water-limited conditions, fix more carbon, and transpire less water. There is natural genetic variability for WUE and this can be used for screening germplasm to identify better genotypes. Evaporative demand is the driving force for water loss and WUE can be improved by increasing transpiration efficiency (TE), alteration in crop phenology, increased carbon fixation, and increased harvest index (HI) by greater partitioning of assimilates into harvestable product. Modification of root architecture, leaf morphology, and stomata conductance are important targets for developing cultivars with improved WUE. Drought tolerance is closely associated with WUE and factors contributing to maintenance of metabolic function under water-limited conditions contribute to improved WUE. Studies on *Arabidopsis* have contributed to significant advances in our understanding of WUE and drought tolerance. The use of genetic engineering and genomic tools has allowed for the incorporation of identified genetic factors for improving WUE and drought tolerance traits and will be vital to the development of new *Brassica* cultivars. The carbon fixation machinery, a vital component in yield, will require adjustments to deal with anticipated water deficits in order to take advantage of increases in atmospheric carbon dioxide as a result of climate change. Manipulation of assimilate partitioning and selection of genotypes

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with capacity to store water-soluble carbohydrates in stems that can be remobilized to harvestable structures are targets for improving WUE. To maintain high yields of *Brassica* crops with economic use of water will require substantial increases in our understanding of the biological processes associated with growth under water-limited conditions. The availability of potential gene targets from the present and future discoveries including rapid advances coming from application of genomic technologies may provide a valuable resource base for development of superior WUE *Brassica* crops in the coming years.

50.1 Introduction

The genus *Brassica* contains a number of species of agricultural importance that are widely grown as oilseeds, vegetables, and forages and that are well adapted to a range of climatic conditions. *Brassica* oilseed crops are an important component of the global vegetable oil market and are valuable sources of condiments and vegetable protein. At present, canola quality *Brassica* oilseed crops are grown on over 25 million ha worldwide [1] with *Brassica napus* having the largest area. *B. oleracea* L. contains a number of varieties that are grown for consumption as vegetables [2]. In addition to use as a food, there are a number of secondary metabolites that are of medicinal and nutraceutical importance [3]. A number of *Brassica* species, including *B. napus* spp. *biennis* L. (forage rape) and *B. rapa* L. (turnip) are of importance as forages [4]. Increased yield is generally the main focus of improvement of agricultural crops and with the projected increase in demand for food and the use of food crops for energy and industrial feedstock, increased yield is now an imperative. *Brassica* crops can be a vital component of any strategy aimed at ensuring food and energy security. Consequently, there must be efficient use of input resources and maximization of yield. In other words, over all plant performance must be improved. While yield is a function of the genetic component of the plant, there are a number of environmental constraints including biotic and abiotic stress, which affect the final outcome. For any biological system, water is a vital component and in the case of agriculture, about 70% of the available freshwater is used in crop production [5–7]. It is now recognized that increased urbanization and the impact of climate change on water use and precipitation, will likely reduce the amount of water available for agriculture [8, 9]. A significant portion of agricultural water use is in crop production [10], and to increase yield, plants must be more efficient in water use. In this chapter, the focus will be on the status of water use efficiency in *Brassica* crop species and ways to enhance this efficiency without compromising yield. We will draw liberally from published work on water use efficiency (WUE) in other crop species such as cereals. Water use efficiency is interrelated to plant performance under water-limited conditions and this necessitates discussion of this topic in the context of drought tolerance and plant growth under water-limited conditions.

50.2 Yield

There is now the realization that yield of all crops must be increased to meet growing demand and this must be achieved in an environmentally sustainable manner. In the case of *Brassica* crops, like other crop plants, yield is the portion of biomass that is partitioned into seed, leaf, stem, root, or floral buds. To realize the genetic potential of a crop, as reflected in yield, environmental factors that negatively affect yield must be controlled. The major limitation to crop yield is water availability or drought [11]. To improve yield, with anticipated decline in water availability for agriculture, plants must be equipped with built-in genetic capacity to use water more efficiently. In cases where water is the limiting factor to plant growth, crop yield (CY) is a function of WU × WUE × HI (WU = total water use, WUE = water use efficiency, and HI = harvest index (harvestable biomass/total biomass)) [12]. Assuming these three terms are independent, an increase in any one is reflected in CY increase [13, 14].

50.3 Water Use Efficiency

The term water use efficiency is used to detail the amount of water per unit of biomass produced. This ratio of biomass to evapotranspiration is generally expressed as WUE (biomass) = (TE/1) + (E_s/T). TE is transpiration efficiency, which is the dry matter/transpiration, E_s is the water lost to evaporation from the soil, and T is transpiration by the crop [15]. This means that to increase WUE, TE must be increased or E_s reduced. In general, there are several management strategies to reduce soil water evaporation such as increasing ground cover, improving plant vigor, and optimizing nutrient status of the plant [15]. Improving transpiration efficiency, TE, can also be achieved through management, as less water will be transpired by crops that accumulate maximum biomass under cooler conditions. This is the case as the main driver for transpirational water loss is the saturated water vapor deficit. Breeding for plants with increased transpiration efficiency is desirable as this trait is under genetic control [16–18]. Plants with pubescent leaves are likely to transpire less water. Also plants with thick cuticles generally have reduced leaf water loss. Genetic variation in cuticular water loss has been reported [18, 19], and selection for genotypes with reduced water loss by this route may improve overall TE. Selection for improved TE may be hampered by the accuracy with which this can be measured efficiently and conveniently. The close relationship between $C^{13}O_2$ and $C^{12}O_2$ discrimination during photosynthetic carbon assimilation and TE was established and is considered a reliable breeding tool for selecting genotypes with high TE, at least in C3 plants [20, 21]. This technique has been used effectively in selecting wheat genotypes with high TE [22]. The advantages and limitations of this technique have been discussed [22, 23].

50.4
Drought Tolerance and WUE

Water availability is perhaps the most important factor limiting plant growth and crop yield. Given the prospect of future drought and water scarcity for crop production, as a consequence of climate change, the crop plant growth will likely occur under suboptimal water condition. Compared to other abiotic stresses affecting plant growth, drought is the one with the most far-reaching negative consequences [23]. Thus, a fundamental knowledge of how plants sense and develop mechanisms to adapt to water-limiting conditions is an imperative. Although WUE in crop production has received considerable attention, there is a widely held view that it is effective use of water, EUW, and not WUE, which is important in over all water conservation [24–26]. This concept is regarded as the important aspect of achieving drought tolerance. This involves assessment of critical periods of crop growth that are sensitive to water stress. Although modification of agronomic practices such as a modification of crop phenology and deficit irrigation through partial root zone drying, PRD, among others, genetic manipulation using transgenics may be required to address the maintenance of crop yield under water-limited conditions. Partial root zone drying may be useful in improving WUE as it probably maintains plant water status through hormonal signals that regulate stomatal function [27]. Two recent publications have highlighted the importance of farming system management in association with plant genotype to improved productivity in water-limited environments [26, 28]. Under conditions of water limitation, the main concern for improved crop yield will be maintenance of growth and biomass accumulation and effective strategies must be directed to this end. Modification in root and shoot traits may be essential for maintaining plant water status under drought conditions. For example, deep root system with increased lateral branching will be able to tap soil water at greater depths. This deep root system may also contribute to increased nutrient uptake resulting in more growth and biomass accumulation and consequently increased WUE. Breeding for drought tolerance through modification of root traits is desirable, but there are technical difficulties in accurately defining the phenotypes, as roots of crop plants are largely inaccessible for convenient observations. The use of closely or tightly linked molecular markers may assist in introgression of desirable WUE traits from wide germplasm [29]. Stomatal and epidermal conductances are important shoot traits relevant to drought tolerance and WUE as about 90% of water uptake is lost by transpiration [30]. Therefore, minimizing this loss is vital. However, gas exchange must be optimized to allow carbon assimilation without excessive water loss. Breeding for leaf pubescence density will likely increase leaf reflectance causing lower leaf temperature and lower water loss at high irradiance [30]. This may also increase leaf boundary layer resistance and enhance photosynthesis [31] contributing to increased WUE. Under conditions of drought, plant cells may maintain turgor and growth by active accumulation of solutes, a process called osmotic adjustment (OA). These solutes can be carbohydrates, amino acids, or sugar acids [32]. In soybean, a positive correlation was

found between the rate of decline in relative water content and relative OA [33]. The effect of OA on yield is debatable [34–36], but it may allow maintenance of metabolic machinery for growth resumption under favorable water conditions. However, a positive correlation between seed yield and OA was observed in *Brassica* species [37, 38]. There was also a close association between stomatal conductance, canopy temperature, and OA in the same species [39, 40]. These findings suggest that species differences or the type of osmolyte may affect physiological and biochemical responses to OA. As a strategy for maintaining turgor under conditions of water deficit by using OA, there may be a reduction in biomass as significant amount of energy can be expended in the production of osmolytes.

50.5 Stomatal Water Loss

Stomata are vital to carbon assimilation, and because CO_2 uptake is tied to water vapor loss an important aspect of WUE is to strike a balance between minimizing water loss while maximizing CO_2 assimilation. Therefore, there must a deeper understanding of the regulatory factors that control transpiration efficiency (the ratio of CO_2 assimilated to water transpired). ABA is well known to function in the regulation of stomatal aperture [41], and while this hormone is a factor in transpiration efficiency, there are several others such as cell wall composition, G protein, GPA1, RD20, a stress-inducible Caleosin, and the ERECTA gene product that are reported to be involved in the regulation of transpiration efficiency [15, 42–45]. The discovery and characterization of the ERECTA gene has potential benefits for transpiration efficiency improvement in crop plants as it affects both photosynthesis and transpiration [15]. Similarly, expression of the gene HARDY in rice significantly improved WUE through root and shoot modification [46]. There is natural genetic variation in transpiration efficiency in several plant species including Brassicas and this could be exploited to improve WUE in *Brassica* crops. There are other factors such as hydrogen sulfide and the enzyme myrosinase, which appear to be involved as signaling components in hormone-induced stomatal closure and may be important to TE [47, 48]. Stomatal density regulation is also a factor in TE as there may be an optimum density for improved WUE [49]. There is also evidence indicating an ABA-independent stomatal aperture regulatory pathway [50], which underscores the complexity of the stomatal regulatory process. Understanding the factors controlling stomatal density will also be vital to identifying genotypes with improved transpiration efficiency [51, 52].

In addition to stomatal water loss, epidermal water loss through the cuticle can be significant to overall TE, and in cotton WUE was negatively correlated with leaf epidermal conductance [18]. Also, cuticular wax composition may influence the transpiration barrier properties of the cuticle [53]. Consequently, screening *Brassica* genotypes for variation in cuticular wax composition and epidermal water loss should be considered while breeding for improved WUE.

50.6
Water Acquisition

Root characteristics will largely determine the efficiency of water uptake under water-limited conditions and root size influences both yield and WUE [54]. Plants with deep root system will be able to tap water from deeper soil layers, compared to those with shallow root system. Root length and degree of branching are characteristics that should be considered and examined in breeding for water acquisition traits. As outlined in Section 50.4, it is technically difficult to phenotype roots for selection purposes, and the use of molecular tagging and measurement of canopy temperature are suggested as alternatives for selecting root-related traits [55, 56]. The development of a phenotyping platform for root systems should advance selection for root traits [57]. Water uptake can also be enhanced by breeding for preferential root OA to sustain growth and water uptake under deficit conditions. An important aspect of root function in WUE is root–shoot signaling under water deficit conditions. ABA appears to play a role in this process and WUE can be improved by such techniques as root-deficit irrigation (RDI) or partial root drying (PRD) [58]. However, other factors such as hydraulic and pH signaling may be involved [59]. These methods are designed to increase stomatal sensitivity to ABA to effect partial closure and reduced water loss while CO_2 assimilation is maintained. The process is complicated by the influence of other hormones such as ethylene that counteracts the ABA response [58].

50.7
Carbon Assimilation

Improvement in WUE will ultimately depend on net carbon gain through photosynthetic carbon fixation. This means that factors such as internal CO_2 concentration, activity of the main CO_2 fixation enzyme Rubisco, and photorespiratory carbon loss must be optimized [60]. It is generally accepted that C4 plants maintain higher WUE, compared to C3 plants, such as *Brassica* species, as a consequence of the ability to concentrate CO_2 at the site of fixation. As a result, there is interest in expressing C4 carbon fixation pathways in C3 crop plants [61]. Modification of the catalytic activity and specificity of Rubisco have also been suggested [61–64]. While this is a potential target for improving net carbon gain in *Brassica* crop species, due consideration should be given to carbon partitioning into harvestable products as this has a significant impact on photosynthetic efficiency. There is evidence indicating the importance of sucrose transporters, SUTs, in carbon partitioning [65]. These transporters could be manipulated in *Brassica* crop species for enhanced carbon gain. Stem storage and remobilization of water-soluble carbohydrates (WSCs) to reproductive structures during grain filling increased harvest index [66]. *Brassica* genotypes with this characteristic could make a significant contribution to WUE and increased yield. Improving carbon fixation efficiency in *Brassica* oilseed species is vital as these species generally require greater biomass to produce the same amount of storage products as do carbohydrate storage crops. For increased biomass,

consideration must be given to factors contributing to high-yield potential under water-limited conditions. Increased understanding of how plants reorder metabolism to maintain growth under water deficit conditions will be vital to development of drought-tolerant and high-yield cultivars capable of improved efficiency in the use of water. For example, invertases have been shown to play a vital role in carbohydrate metabolism and yield under conditions of stress [42, 67]. This suggests that knowledge of how plants adapt various metabolic processes to maintain growth under unfavorable conditions will be vital to the development of genotypes for efficient use of input resources.

50.8
Importance of other Growth-Limiting Factors to WUE

There are many factors such as adequate supply of nutrients, pests and diseases, soil conditions, temperature, and abiotic stresses that may influence crop performance and WUE. Consequently, the impact of these conditions on the performance of *Brassica* crops must be taken into account in the development of cultivars with improved WUE. Improvement in nitrogen nutrition increased WUE through increased photosynthesis [68, 69]. Improved nitrogen nutrition may also be involved in ABA-induced root–shoot signaling and stomatal regulation [70]. Soil microflora may have a positive effect on WUE by both improving nutrient acquisition and providing signal molecules for stomatal regulation [58]. It is likely that soil mycorrhizae may contribute to WUE and this is an area that should be explored as it may offer complimentary and synergistic improvements.

When the potential consequences of climate change to crop productivity and agriculture in general are taken into account, it is clear that the sustainability will depend on the use of advance technologies to increase productivity. Breeding for increased productivity must include consideration of efficient use of all input resources [71]. There is little debate that the significant advances in our knowledge of biology in general, but specifically microbial and plant biology, should be the platform for addressing sustainability of agricultural systems. To improve WUE in *Brassica* crop species will require sustained advances in our knowledge of how these plants function under water-limited conditions and their interaction with other environmental stresses. The status of our knowledge of plant biology has improved our understanding of gene expression and genetic networks responsive to drought tolerance and water deficit [72–75]. There is a significant body of knowledge available on the physiological and biochemical aspects of plant response to drought, and the role of ABA in these processes has been advanced [76–78]. However, the mechanisms of action of these gene products remain to be established [79]. The application of genetic engineering tools has resulted in the development of transgenic crop plants expressing genes conferring tolerance to drought and increased WUE [76]. Some of these genes exhibit undesirable side effects, which may limit broader usage. However, regulated expression may circumvent these negative outcomes. Wild *Brassica* species may contain genes conferring improved WUE and drought toler-

ance. These can be introgressed into crop species by conventional breeding. However, genetic engineering offers a better option for the development of crops with such traits as access for desirable genes is potentially unlimited. This also offers an excellent platform for increasing our knowledge of gene function in a variety of genetic backgrounds. The overall aim of drought tolerance and efficiency of water use should be to maximize yield under water limited conditions. To this end, it is important to know how plants maintain homeostasis and metabolic activity under such conditions. To gain this knowledge, gene function cannot be viewed in isolation but must be viewed in the context of interacting genetic and metabolic networks in a well-integrated comprehensive systems approach [80, 81].

50.9
Water Use Efficiency in *Brassica* Species

Brassica crop species are adapted to a range of environments and like in other crop species yield is heavily influenced by water availability. Many areas of *Brassica* crop cultivation are drought prone and to meet increasing demand for *Brassica* products, it will be necessary to expand cultivation into less favorable areas. Therefore, effective water use for yield maintenance is vitally important. In spite of the importance of these species, there are not many reported studies of WUE. In a comparison of WUE and stomatal conductance in *Moricandia* and *Brassica* species, McVetty *et al.* [82] attributed lower WUE in the latter species to higher stomatal conductance. Studies with *B. oleracea* revealed a number of quantitative trait loci (QTL) for variation in leaf conductance and photosynthetic assimilation rate [83]. These findings may be useful in breeding for improved WUE [82]. Variation in tolerance to drought, attributable to differential osmotic adjustment, among *Brassica* species has been reported [84]. There are genotypic differences in response to drought stress, which may be related to OA [84–86]. These studies indicate availability of a rich source of genetic variation for drought tolerance and WUE in *Brassica* species that can be exploited in breeding for both drought tolerance and WUE.

50.10
Conventional Breeding for WUE in *Brassica* Crop Species

This invariably involves breeding for drought tolerance and growth under water-limiting conditions. With the availability of germplasm, conventional breeding can make a significant contribution to improvement in WUE and drought tolerance. Physiological and morphological traits related to WUE can be identified and their inheritance determined. Carbon isotope discrimination (CID), which measures the ratio of $^{13}C/^{12}C$ in the plant tissues compared to the air, is an indirect measure of WUE and can be used to detect genetic variation for TE in plants. This could provide an effective tool for screening the germplasm. Screening can also be done by measuring leaf ash content, LASH, and K content [87, 88]. Ash content is generally

negatively associated with WUE if a constant concentration of minerals in the transpiration stream is maintained. Since this is likely to be affected by environmental conditions, the accuracy of genotypic comparison, they should be grown in the same environment. This method has been used to identify QTL for WUE in soybean [87]. A potentially promising area of study is to screen the germplasm for differences in root architecture in relation to WUE. This is an often underexplored area of research that could improve both yield and WUE [88–90]. Improving root architecture and size will be essential to ensuring adequate transpiration during water deficit, which will have a positive influence on productivity [91–93]. QTL for root architecture in rice have been identified and their effect on yield determined [88]. Application of similar approaches in Brassicas may contribute to new potential gene targets for WUE traits in *Brassica* species.

50.11
Unique Challenges to Breeding for WUE in *Brassica* Crop Species

As a consequence of the diversity of *Brassica* crop species, where the harvestable products include oilseeds, leaves, stems, florets, flower buds, axillary buds, and tubers, breeding for WUE is naturally challenging. This is in contrast to major grain crops such as rice, wheat, and maize where the grain is generally the product at harvest. Therefore, WUE strategies such as increasing sensitivity of stomates to ABA may be unsuitable for leafy *Brassica* crops as leaf growth may be compromised [58]. Larger leaves will usually transpire more water, which means that for leafy *Brassica* crops increased WUE should be by means that do not compromise leaf growth. Increasing photosynthetic efficiency and enhancing crop development under conditions where evaporative demands are lower should improve WUE in these crops.

50.12
Engineering Drought Tolerance and WUE in *Brassica* Crops

Although conventional breeding will undoubtedly contribute to improvement in WUE, there are substantial benefits to be derived from the application of a biotechnological approach. By this approach, there is no limitation to the source of genes that can be evaluated. In contrast, conventional breeding is limited by the compatibility of germplasm with the genotype under improvement. This, in effect, widens the gene pool for improvement in WUE and drought tolerance. Another benefit of this approach is the potential to gain valuable information on gene function after expression in the desired background. Given the importance of abiotic stress to plant growth, the literature on attempts to engineer stress tolerance in plants is voluminous and there are a number of approaches for the identification of genes involved in abiotic stress [94, 95]. The model plant *Arabidopsis* has been an invaluable tool in advancing our knowledge of plant biology in general and specifically some fundamental aspects of abiotic stress responses. This topic has been reviewed

recently [94] and will not be further discussed here. The ability to identify genes for specific functions and to express them in heterologous systems through genetic engineering technology has revolutionized plant biology. Genes conferring tolerance to abiotic stresses have been evaluated in the model species *Arabidopsis* and in other plants [96–100]. The attractiveness of this technology is that we can introduce only the genes for specific traits into an elite variety without carrying along other potential undesirable genes, as is the case with conventional breeding. In that case, several rounds of backcrossing are normally required to remove the unwanted genes. An excellent example is the identification and introduction of the transcription factor NFYB2 into maize [101]. This conferred drought tolerance, WUE, and increased yield when tested under water-limited field conditions. There are a number of cases where drought tolerance, WUE, have been achieved by genetic approaches including engineering of functional and regulatory proteins, and enzymes for production of osmolytes and osmoprotectants [76, 79]. Many of these, though at the proof-of-concept stage, still show promise for incorporation into crop species to develop superior WUE traits.

In many environments where *Brassica* crop species are grown yield will be heavily influenced by water availability. Therefore, effective water use to sustain yield is vitally important. In spite of the importance of these species, there are not many studies on WUE.

Success in improving WUE and growth under water-limited conditions depends on an understanding of plant physiology and metabolism under conditions of water limitation [102]. Since the economics of water use is a function of carbon assimilation per unit of water used, efficiency can be obtained by addressing factors limiting carbon assimilation along with those controlling water losses. It is well established that a major consequence of reduced plant water status is impairment in the photosynthetic machinery [79, 102]. This is the result of reduction in internal CO_2 levels as stomates close in response not only to water deficit but also to disruption in oxidative homeostasis alterations in cellular metabolism. To maintain photosynthetic carbon assimilation, plants must be equipped with enzymes to alleviate oxidative stress. About 90% of the water absorbed by plants is lost in transpiration. This means that WUE can be improved by regulating stomatal water loss. The plant hormone ABA is a major factor in the regulation of stomatal aperture and reduction in transpirational water loss [41]. By uncovering the molecular components regulating this ABA response, it was possible to engineer drought tolerance in canola (*B. napus* L.) [103–105]. Negative regulation of ABA via downregulation of ERA1 has been reported to both enhance drought tolerance and maintain productivity in *Brassica* [104, 105]. Drought tolerance and improved WUE was also reported for canola plants expressing a gene for lipid metabolism, PtdIns-PLC2, [106]. In another study, transgenic canola plants expressing poly (ADP-ribose) polymerase exhibited lower reactive oxygen species (ROS) and displayed drought tolerance [107]. Transgenic canola plants with wheat mitochondrial Mn superoxide dismutase (Mn SOD3.1) exhibited increased vigor and tolerance to abiotic stress, including drought [108]. Similarly, the overexpression of LEA proteins has been shown to impart drought tolerance to *Brassica* seedlings [109]. However, the *caveat* is that it has yet to be

determined that the overexpression of abiotic stress-tolerant genes or transcription factors regulating these genes does not have a negative overall yield crop productivity under field conditions.

50.13
Prospects of Improving WUE in *Brassica* Crops

There is general agreement that crop productivity must be increased to meet increasing global demand [110]. It is clear that this must be achieved by a more efficient use of input resources. This requires development of crop cultivars with enough genetic capacity for high yields with minimum input. Access to desirable genes from natural variation in wild relatives of *Brassica* species will enhance development of cultivars with improved WUE. However, the application of genomics and tools of biotechnology will be vital to this activity [111]. Consideration must also be given to crop interaction with biotic and abiotic stresses that impact yield in the sense of acquiring fundamental knowledge of physiological and biochemical mechanisms underlying adaptation to stress [112, 113]. Climate change is predicted to drastically alter crop growth environment in a largely unpredictable manner, which is a major challenge to future crop yields [62, 63]. Water availability is perhaps the most limiting factor to crop growth and yield and with the prospect of further decline in this resource, as a consequence of climate change, its efficient use is paramount. For *Brassica* crops to continue to play a significant role in future food, feed, and energy security, there is need to improve WUE and drought tolerance in these species and to extract higher yields per unit of water used. While there are a number of measures proposed to conserve soil water [24, 58, 66, 113], significant research emphasis should be on the efficiency with which water is used for biomass generation in *Brassica* crops. The identification and characterization of two genes, ERECTA and HARDY [15, 46] that enhanced WUE through multiple effects on plant morphology, should be useful in the development of WUE *Brassica* cultivars. Root–shoot signaling is emerging as an important aspect of stomatal aperture regulation [114, 115], and further advances in this area should benefit plant breeding efforts aimed at the development of WUE genotypes. Regulation of stomatal water loss, improved root water acquisition, and finding ways to effect greater efficiency of carbon assimilation and partitioning into harvestable products under water-limited conditions, are likely to be keys to WUE in *Brassica* species.

Though significant progress has been made in the identification of several genetic factors implicated in WUE in plants, it is likely that many others involved remain to be identified. Rapid advances in genomic technologies offer complementary approaches for performing global gene expression analyses to identify new factors associated with WUE in model plants such as *Arabidopsis* and closely related *Brassica* crop species. New gene discoveries coupled with insights into their function and regulation will expand potential new targets for improvement of WUE in *Brassica* species.

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References

1 Commodity Research Bureau (2005) *CRB Commodity Year Book*, Commodity Research Bureau, Jersey City, NJ.
2 King, G.J. (1990) *Euphytica*, **50**, 97–112.
3 Mithen, R.F., Dekker, M., Verkerk, R. *et al.* (2000) *J. Sci. Food Agric.*, **80**, 967–984.
4 McCartney, D., Fraser, J., and Ohama, A. (2009) *Can. J. Anim. Sci.*, **89**, 431–440.
5 Pennisi, E. (2008) *Sci.*, **320**, 171–173.
6 Cominelli, E., Galbiati, M., Tonelli, C. *et al.* (2009) *EMBO Rep.*, **10**, 671–676.
7 Condon, A.G., Richards, R.A., Rebetzke, G.J. *et al.* (2004) *J. Exp. Bot.*, **55**, 2447–2460.
8 Gornall, J., Betts, R., Burke, E. *et al.* (2010) *Philo. Trans. R. Soc. B.*, **365**, 2973–2989.
9 Strzepek, K. and Boehlert, B. (2010) *Phil. Trans. R. Soc. B*, **365**, 2927–2940.
10 Morison, J.I.L., Baker, N.R., Mullineaux, P.M. *et al.* (2008) *Phil. Trans. R. Soc. B*, **363**, 639–658.
11 Inostroza, L. and Acuna, H. (2010) *Plant Breed.*, **129**, 700–706.
12 Passioura, J.B. (1977) *J. Aust. Inst. Agric. Sci.*, **43**, 117–120.
13 Araus, J.L., Slafer, G.A., Reynolds, M. *et al.* (2002) *Ann. Bot.*, **89**, 925–940.
14 Richards, R.A., Rebetzke, G.J., Condon, A.G. *et al.* (2002) *Crop Sci.*, **42**, 111–121.
15 Masle, J., Gilmore, S.R., and Farquhar, D. (2005) *Nature*, **436**, 866–870.
16 Bunce, J.A. (2010) *Crop Sci.*, **50**, 1409–1413.
17 Fish, D.A. and Earl, H.J. (2009) *Crop Sci.*, **49**, 1409–1415.
18 Clarke, J.M. and McCaig, T.N. (1982) *Can J. Plant Sci.*, **62**, 571–578.
19 Farquhar, G.D. and Richards, R.A. (1984) *Aust. J. Plant Physiol.*, **11**, 539–552.
20 Condon, A.G., Richards, R.A., and Farquhar, G.D. (1992) *Aust. J. Agric. Res.*, **43**, 935–947.
21 Condon, A.G. and Richards, R.A. (1992) *Aust. J. Agric. Res.*, **43**, 921–934.
22 Masle, J., Farquhar, G.D., and Wong, S.C. (1992) *Aust. J. Plant Physiol.*, **19**, 709–721.
23 Chaves, M. and Davies, B. (2010) *Funct. Plant Biol.*, **37**, iii–vi.
24 Passioura, J. (2007) *J. Exp. Bot.*, **58**, 113–117.
25 Passioura, J. (2006) *Agric. Water Manag.*, **80**, 176–196.
26 Blum, A. (2009) *Field Crops Res.*, **112**, 119–123.
27 Dodd, I.C. (2009) *J. Exp. Bot.*, **60**, 2454–2459.
28 Kirkegaard, J.A. and Hunt, J.R. (2010) *J. Exp. Bot.*, **61**, 4129–4143.
29 Vuong, T.V., Wu, X., Pathan, M.M.S. *et al.* (2007) *Genomics-Assisted Crop Improvement* (eds R.K. Varshney and R. Tuberosa), Springer, New York, pp. 243–279.
30 Garay, A.E. and Wilhelm, W.W. (1983) *Agron. J.*, **75**, 973–977.
31 Specht, J.E. and Williams, J.H. (1985) *World Soybean Research Confererence III* (ed. R. Shibles), Westview Press, pp. 468–475.
32 Rontein, D., Basset, G., and Hanson, A. (2002) *Metab. Eng.*, **4**, 49–56.
33 James, A.T., Lawn, R.J., and Cooper, M. (2008) *Aust. J. Agric. Res.*, **59**, 656–669.
34 Munns, R. (1988) *Aust. J. Plant Physiol.*, **15**, 717–726.
35 Morgan, J.M. and Condon, A.C. (1986) *Aust. J. Plant Physiol.*, **13**, 523–532.
36 Passioura, J. (1986) *Aust. J. Plant Physiol.*, **13**, 191–201.
37 Kumar, A., Singh, D.P., and Singh, P. (1987) *J. Agric. Sci.*, **109**, 615–618.
38 Singh, D.P., Kumar, A., Singh, P. *et al.* (1990) Proceedings of the International Congress on Plant Physiology, New Delhi, India, Feb. 15–20 1987, pp. 841–848.

39 Kumar, A., Singh, P., Singh, D.P. *et al.* (1984) *Ann. Bot.*, **54**, 537–541.

40 Singh, D.P., Singh, P., Kumar, A. *et al.* (1985) *Ann. Bot.*, **56**, 815–820.

41 Addicott, F.T. and Lyon, J.L. (1969) *Annu. Rev. Plant Physiol.*, **20**, 139–164.

42 Ruan, Y.-L., Jin, Y., Li, G.-J. *et al.* (2010) *Mol. Plant.*, **3**, 942–955.

43 Laing, Y.-K., Xie, X., Lindsay, S.E. *et al.* (2010) *Plant J.*, **64**, 679–686.

44 Nilson, S. and Assmann, S.M. (2010) *Plant Physiol.*, **152**, 2067–2077.

45 Aubert, Y., Vile, D., Pervent, M. *et al.* (2010) *Plant Cell Physiol.*, **51**, 1975–1987.

46 Karaba, A., Dixit, S., Greco, R. *et al.* (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 15270–15275.

47 Garcia-Mata, C. and Lamattina, L. (2010) *New Phytol.*, **188**, 977–984.

48 Suhita, D., Raghavendra, A.S., Kwak, J.M. *et al.* (2004) *Plant Physiol.*, **134**, 1536–1545.

49 Wang, Y., Chen, X., and Xiang, C.-B. (2007) *J. Integr. Plant Biol.*, **49**, 1435–1444.

50 Song, X.-J. and Matsuoka, M. (2009) *Genes Dev.*, **23**, 1709–1713.

51 Kondo, T., Kajita, R., Miyazaki, A. *et al.* (2010) *Plant Cell. Physiol.*, **51**, 1–8.

52 Sugano, S.S., Shimida, T., Imai, Y. *et al.* (2010) *Nature*, **463**, 241–246.

53 Vogg, G., Fisher, S., Leide, J. *et al.* (2004) *J. Exp. Bot.*, **55**, 1401–1410.

54 Chloupek, O., Dostal, V., Streda, T. *et al.* (2010) *Plant Breed.*, **129**, 630–636.

55 Manalavan, L.P., Guttikonka, S.K., Tran, L.P. *et al.* (2009) *Plant Cell Physiol.*, **50**, 1260–1276.

56 Reynolds, M. and Tuberosa, R. (2008) *Curr. Opin. Plant Biol.*, **11**, 171–179.

57 Iyver-Pascuzzi, A.S., Symonova, O., Mileyko, Y. *et al.* (2010) *Plant Physiol.*, **152**, 1148–1157.

58 Wilkinson, S. and Hartung, W. (2009) *J. Exp. Bot.*, **60**, 1885–1891.

59 Ren, H., Wei, K., Jia, W. *et al.* (2007) *J. Integr. Plant Biol.*, **49**, 1410–1420.

60 Raines, C.A. (2011) *Plant Physiol.*, **155**, 36–42.

61 Zhu, X.G., Long, S.P., and Ort, D.R. (2010) *Annu. Rev. Plant Biol.*, **61**, 235–261.

62 Ainsworth, E.A., Rogers, A., and Leaky, A.D.B. (2008) *Plant Physiol.*, **147**, 13–19.

63 Long, S.P. and Ort, D.R. (2010) *Curr. Opin. Plant Biol.*, **13**, 241–248.

64 Price, G.D., Badger, M.A., and von Caemmere, S. (2011) *Plant Physiol.*, **155**, 20–26.

65 Braun, D.M. and Slewinski, T.L. (2009) *Plant Physiol.*, **149**, 71–81.

66 Richards, R.A., Rebetzke, G.J., Watt, M. *et al.* (2010) *Funct. Plant Biol.*, **37**, 85–97.

67 Boyer, J.S. (2010) *J. Exp. Bot.*, **61**, 3493–3497.

68 Wang, Y., Liu, F., Andersen, M.M. *et al.* (2010) *Funct. Plant Biol.*, **37**, 175–182.

69 Cabrera-Bosquet, L., Molero., G., Nogues, S. *et al.* (2009) *J. Exp. Bot.*, **60**, 1633–1644.

70 Wilkinson, S., Bacon, M.A., and Davies, W.J. (2007) *J. Exp. Bot.*, **58**, 1705–1716.

71 Witcombe, J.R., Hollington, P.A., Howarth, C.J. *et al.* (2008) *Phil. Trans. R. Soc. B.*, **636**, 703–716.

72 Shinozaki, K. and Yamaguchi-Shinozaki, K. (2007) *J. Exp. Bot.*, **58**, 221–227.

73 Valliyodan, B. and Nguyen, H.T. (2006) *Curr. Opin. Plant Biol.*, **9**, 189–195.

74 Bray, E. (2004) *J. Exp. Bot.*, **55**, 2331–2341.

75 Umezawa, T., Fujita, M., Fujita, Y. *et al.* (2006) *Curr. Opin. Plant Biotechnol.*, **17**, 113–122.

76 Yang, S., Vanderbeld, B., Wan, J. *et al.* (2010) *Mol. Plant*, **3**, 469–490.

77 Zou, J.-J., Wei, F.-J., Wang, C. *et al.* (2010) *Plant Physiol.*, **154**, 1232–1243.

78 Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R. *et al.* (2010) *Annu. Rev. Plant Biol.*, **61**, 651–679.

79 Chaves, M.M. and Oliveira, M.M. (2004) *J. Exp. Bot.*, **55**, 2365–2384.

80 Yuan, J.S., Galbraith, D.W., Dai, S.Y. *et al.* (2008) *Trends Plant Sci.*, **13**, 165–171.

81 Sweetlove, L.J. and Fernie, A.R. (2005) *New Phytol.*, **168**, 9–24.

82 McVetty, P.B.E., Austin, R.B., and Morgan, C.L. (1989) *Ann. Bot.*, **64**, 87–94.

83 Hall, N.M., Griffiths, H., Corlett, J.A. *et al.* (2005) *Plant Breed.*, **124**, 557–564.

84 Ashraf, M. and Mehmood, S. (1990) *Environ. Exp. Bot.*, **30**, 93–100.

85 Kumar, A. and Singh, D.P. (1998) *Ann. Bot.*, **81**, 413–420.

86 Kumar, A. and Elston, J. (1992) *Ann. Bot.*, **70**, 3–9.

87 Mian, M.A.R., Mailey, M.A., and Ashley, D.A. (1996) *Crop Sci.*, **44**, 1252–1257.

88 Collins, N.C., Tardieu, F., and Tuberosa, R. (2008) *Plant Physiol.*, **147**, 469–486.

89 MacMillan, K., Emrich, K., Piepho, H.P. *et al.* (2006) *Theor. Appl. Genet.*, **113**, 977–986.

90 Steele, K.A., Virk, D.S., Kumar, R. *et al.* (2007) *Field Crops Res.*, **101**, 190–186.

91 Den Herder, G., Van Isterdael, G., Beeckman, T. *et al.* (2010) *Trends Plant Sci.*, **15**, 600–607.

92 Lynch, J. (1995) *Plant Physiol.*, **109**, 7–13.

93 Hammer, G.L., Dong, Z., Mclean, G. *et al.* (2009) *Crop Sci.*, **49**, 299–312.

94 Papdi, C., Joseph, M.P., Salmo, I.P. *et al.* (2009) *Funct. Plant Bol.*, **36**, 696–720.

95 Takeda, S. and Matsuoka, M. (2008) *Nat. Rev. Genet.*, **9**, 444–457.

96 McKersie, B.D., Bowley, S.R., Harjanto, E. *et al.* (1996) *Plant Physiol.*, **111**, 1177–1181.

97 Xu, D., Duan, X., Wang, B. *et al.* (1996) *Plant Physiol.*, **110**, 249–257.

98 Pilon-Smits, E.A., Terry, N., Sears, T. *et al.* (1998) *J. Plant physiol.*, **152**, 525–632.

99 Garg, A.K., Kim, J.-K., Owens, T.G. *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 15898–15903.

100 Saijo, Y., Hata, S., Kyozuka, J. *et al.* (2000) *Plant J.*, **23**, 319–327.

101 Nelson, D.E., Repetti, P.P., Adams, T.R. *et al.* (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 16450–16455.

102 Chaves, M.M., Maroco, J.P., and Pereira, J.S. (2003) *Funct. Plant. Biol.*, **30**, 239–264.

103 Wan, J., Griffiths, R., Ying, J. *et al.* (2009) *Crop Sci.*, **49**, 1539–1554.

104 Wang, Y., Beaith, M., Chalifoux, M. *et al.* (2009) *Mol. Plant.*, **2**, 191–200.

105 Wang, Y., Ying, J., Kuzma, M. *et al.* (2005) *Plant J.*, **43**, 413–424.

106 Georges, F., Das, S., Ray, H. *et al.* (2009) *Plant Cell Environ.*, **32**, 1664–1681.

107 de Block, M., Verduyn, C., De Brouwer, D. *et al.* (2005) *Plant J.*, **41**, 95–106.

108 Gusta, L.V., Benning, N.T., Wu, G. *et al.* (2009) *Mol. Breed.*, **24**, 103–115.

109 Dalal, M., Tayal, D., Chinnusamy, V. *et al.* (2009) *J. Biotechnology.*, **139**, 137–145.

110 Edgerton, M.D. (2009) *Plant Physiol.*, **149**, 7–13.

111 Reynolds, M.P. and Borlaug, N.E. (2006) *J. Agric. Sci.*, **144**, 95–110.

112 Mahajan, S. and Tuteja, N. (2005) *Arch. Biochem. Biophys.*, **444**, 139–158.

113 Neumann, P.M. (2008) *Ann. Bot.*, **101**, 901–907.

114 Loveys, B. and Davies, W.J. (2004) *Water Use Efficiency in Plant Biology* (ed. M. Bacon), Blackwell, Oxford, pp. 113–141.

115 Chaerle, L., Saibo, N., and Der Straeten, D. (2005) *Trends Biotechnol.*, **23**, 308–315.

51
Brassica Crops: Improving Abiotic Stress Tolerance – Current Status and Prospects

Stephen J. Robinson and Isobel A.P. Parkin

A growing awareness of the need to ensure global food security in the face of an expanding population and the uncertainties of a changing climate is driving the demand for enhanced agricultural productivity. Environmental stresses have a significant impact on harvestable yield and alleviating their toll offers opportunities to stabilize and improve crop yields. However, the physiological and genetic responses of plants grown under adverse environments are highly complex and have thwarted improvement strategies. Here, we review our understanding of abiotic stresses and the plant's response to such stresses. We focus on the improvement strategies that have been tested and on the developments in genomics technologies that could facilitate the improvement of stress tolerance in the *Brassica* oilseeds species, which are responsible for generating a large proportion of the world's edible oil.

51.1
Introduction

Humans have been remarkably successful at colonizing the globe with permanent dwellings on every continent. The population is now so numerous that our combined actions can affect Earth's climate. The dramatic explosion of human population is a recent event rising from ~1 billion in 1750 to 3 billion in 1960 and 6 billion by 1999. This exponential growth is anticipated to continue before slowing by ~2050 [1]. This population expansion has been fueled by tremendous increases in agricultural production and productivity along with advances in medical and sanitation practices. Until the beginning of the last century, the energy that sustained human life was obtained solely by photosynthesis capturing energy derived from the sun. This energy was utilized directly through the consumption of crop plants or indirectly from the consumption of animals raised on plant feed. In this way, photosynthesis set

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a limit on the amount of food that could be generated, effectively placing a limit upon population growth.

Technological advances, improvements in agricultural practices, and mechanization improved yields and reduced the labor required for land management, furthering population growth. Agriculture was dramatically transformed during the 1950s, commonly referred to as the Green Revolution, when the provision of optimal growth environments and adapted varieties increased grain yields by 250% [2]. This was the beginning of the industrialization of agriculture with yield increases obtained from the expenditure of fossil fuel-derived energy in the form of fertilizers, pesticides, and hydrocarbon-fueled irrigation. However, as the availability of cheap fossil fuels becomes limited [3, 4] the impact of adverse environmental conditions will be a significant problem to ensuring the sustainability of adequate agricultural output. The development of crops that are better able to tolerate suboptimal growth conditions is essential if the challenges of satisfying the increased food demand are to be addressed.

51.1.1 Introducing the Brassicaceae

The Brassicaceae contains more than 3500 species classified in 350 genera and is one of the ten most economically important plant families. There is enormous variation within the *Brassica* species, and through selective breeding they have been developed as sources of oil, vegetables, mustard condiments, and fodder. Species of particular importance include *Brassica napus* (var. *oleifera*), *B. rapa* (var. *oleifera*), and *B. juncea* as sources of canola and industrial oils. The vegetable crops include broccoli, cauliflower, Brussels sprouts, cabbage, kale (*B. oleracea*), swede or rutabaga (*B. napus*), Chinese cabbage or turnip (*B. rapa*), and radish (*Raphanus sativus*). These are complemented by species grown as condiments that include Indian mustard (*B. juncea*), white mustard (*Sinapis alba*), and horseradish (*Armoracia rusticana*). The cytogenetic relationships among the *Brassica* crop species consist of combinations of three basic genomes aptly described by the triangle of U [5]. The nodes of the triangle are formed by the diploid species *B. rapa* ($2n = 20$, AA), *B. nigra* ($2n = 16$, BB), and *B. oleracea* ($2n = 18$, CC). The amphidipliod species *B. carinata* ($2n = 34$, BBCC), *B. juncea* ($2n = 36$, AABB), and *B. napus* ($2n = 38$, AACC) were each formed by pairwise hybridization among the diploid species (Figure 51.1). Research in the *Brassica* crops has benefited from advances in knowledge and the development of resources for their close relative *Arabidopsis thaliana*, the first plant genome to be fully sequenced [6].

Oilseeds and their products are among the most valuable agricultural commodities in world trade and *Brassica* oilseed species are the most important world sources of vegetable oil after palm and soybean oil. The oilseed *Brassica* crops have undergone a rapid transformation from a marginal break crop 60 years ago to become a major cash crop subsequent to the reduction of both erucic acid and glucosinolate concentration in the seed, now synonymous with canola [7]. Although the dominant *Brassica* oilseed

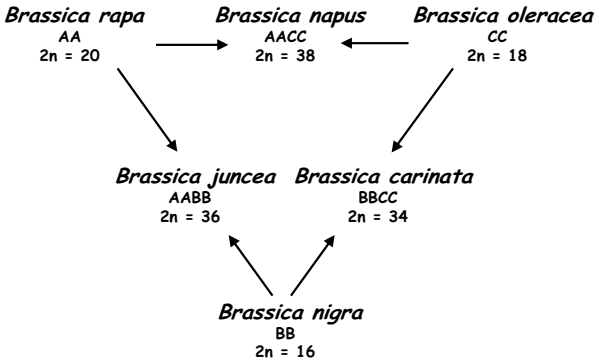

Figure 51.1 Representation of the genetic relationship among the six *Brassica* crop species as suggested by U [5].

is canola, other edible Brassicaceae oilseeds such as mustards, *Crambe*, and *Camelina sativa* constitute important variants. When considering *Brassica* oilseeds alone, an average of 50.8 million tons are produced worldwide each year (2004–2008) and average yields have increased more than twofold over the past 60 years [8]. The countries producing the largest acreages of *Brassica* oilseeds are China, Canada, and India. However, higher yields are obtained from European countries, exceeding twice those obtained in Canada as the more productive biennial varieties can be grown under the favorable European conditions as opposed to the annual varieties adapted to Canadian climates [9].

51.1.2
The Impact of Stress on *Brassica* Yield Factors

The constraint on plant distribution is primarily determined by the combined effects of temperature (Figure 51.2a) and water availability (Figure 51.2b and c) and crop plants tend to be more susceptible to extremes of these conditions. Any factor that results in a decrease in plant growth and yield beneath that predicted by the plant's genotypic potential can be considered a physiological stress. The maximum harvestable yield can be considered the upper limit of a yield potential and the difference between this and the mean harvestable yield represents the losses due to environmental stress. Losses due to abiotic stress are considerable, with estimates exceeding 60% of the yield potential being observed [10]. The local climatic conditions determine the major stresses that have an impact on *Brassica* crops, but the effects of an insufficient water supply, adverse temperatures (including both excessive heat and freezing temperatures), and salinity are frequently responsible for large annual yield losses [10, 11].

The impact of abiotic stresses on *Brassica* crops depends on a number of factors that include type, intensity, and duration of the stress along with the developmental

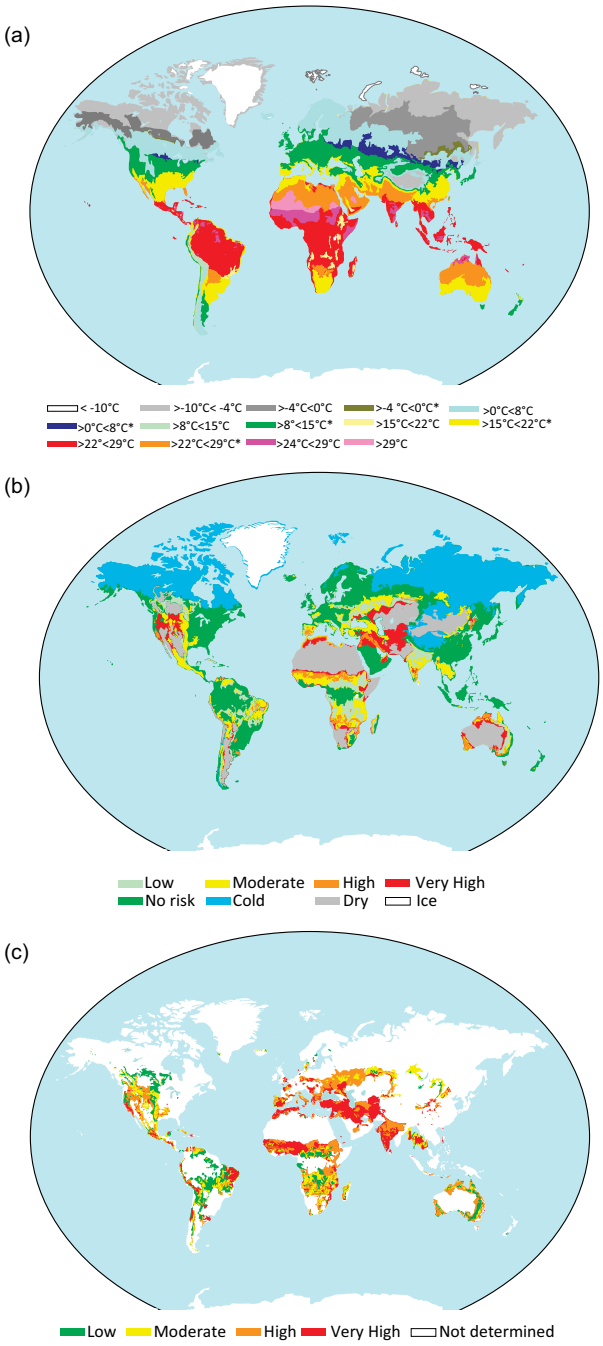

Figure 51.2 Global soil temperature and water availability limit the distribution of plant species and agricultural productivity. Average global soil temperature is presented in (a), the colored key indicates the temperature range; an asterisk denotes a temperature variance in excess of 5 °C. (b) The global soils at risk from desertification. (c) The risk of human-induced desertification. All the data presented are a summary of those produced by the United States Department of Agriculture (http://soils.usda.gov/.)

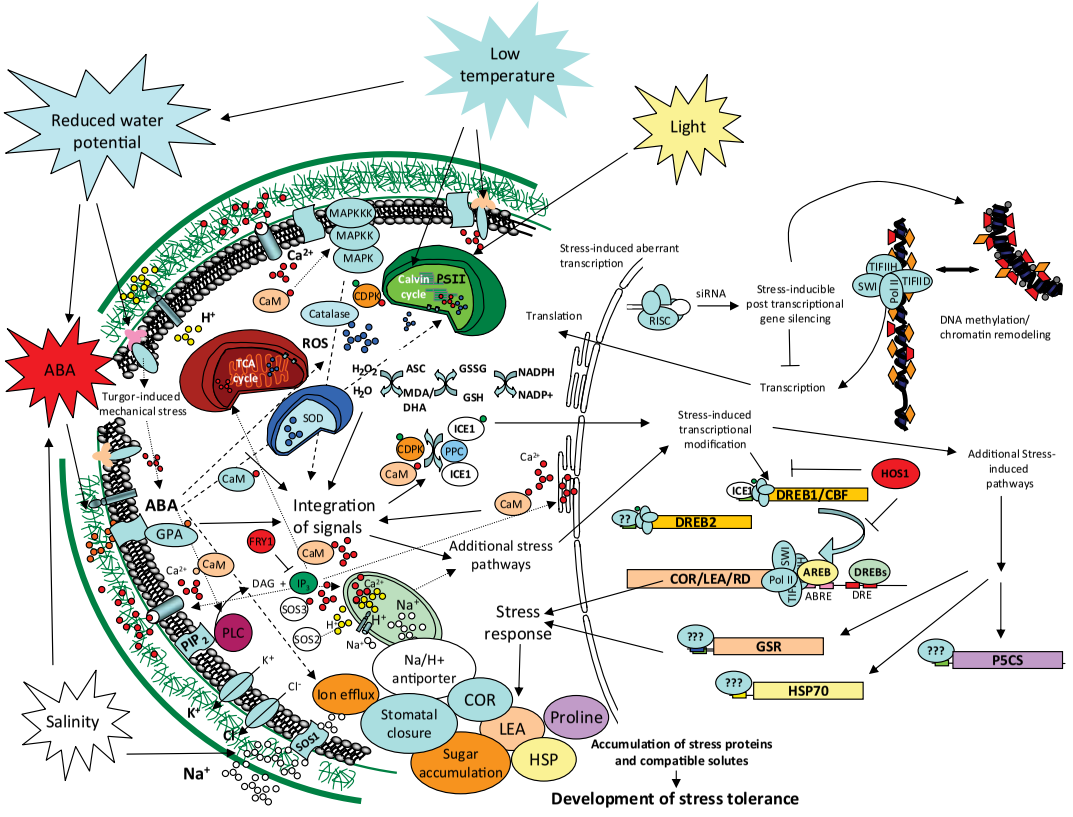

Figure 51.3 Signal transduction pathways involved in abiotic stress within the plant cell. The various abiotic stresses result in changes in membrane fluidity, mechanical perturbations caused by changes in turgor pressure, imbalances in redox potential, and the production of ABA and ROS. Upon stress perception, signals mediated through the action of hormones and secondary messengers (Ca^{2+}, IP_3) result in the activation of phosphorylation/dephosphorylation cascades that result in specific changes to regulatory proteins that activate stress-specific gene expression. Additional transcriptional regulation of abiotic stress can result from changes in DNA methylation patterns along with histone modifications that can be mediated through the action of siRNA. The solid green lines represent the cell wall matrix that sits above the plasma membrane. Integral and peripheral membrane proteins are depicted in blue. The circles represent Ca^{2+} ions (red), H^+ ions (yellow), phosphate groups (green), ROS (blue), Na^+ (white), and methyl groups (gray). Abbreviations: CaM, calmodulin; CDPK, calcium-dependent protein kinase; ASC, ascorbic acid; MDA, monodehydroascorbate; DHA, dehydroascorbate; GSSG, glutathione disulfide; GSH, glutathione; MAPK, MAPKK, and MAPKKK, mitogen-activated protein kinase cascade; PLC, phospholipase C.

stage of the crop exposed to the insult [12]. Among the *Brassica* oilseeds, environmental stresses have the greatest impact on yield during silique formation with the 2 week period post-anthesis being critical for determining the yield obtained [13–15]. During this developmental stage, oilseeds are generally most at risk of being exposed to drought and excess heat stress. Although, for annual crop types, low-temperature stress at the extremes of the growing season, with the occurrence of late-spring or early-autumn frost, can affect yield by reducing plant establishment or the quality and number of seeds per silique, respectively [16]. Arguably, the greatest agronomic impact of frost to oilseed brassicas is the reduction in yield quality resulting from early autumn frosts that damage the developing embryos resulting in chlorophyll retention in the harvested seed [17].

These critical growth stages provide the best targets to increase the stress hardiness of *Brassica* crop plants. The development of crops with targeted stress tolerance in addition to the application of good agricultural practices promises yield stabilization.

51.2
Plants' Response to Abiotic Stress

Many of the physiological adjustments that are utilized by plants to increase their ability to tolerate abiotic stresses have been described in detail for many crop species [12, 18–23] including the *Brassica* crops [24–29]. Although the relationship between many of these physiological adjustments at the cellular level and the stress tolerance in the field are difficult to establish, understanding these processes may yet determine key targets for stress improvement.

51.2.1
Metabolic Adjustments to Stressful Environments

Unique challenges are imposed by each abiotic stress. Perhaps with the exception of flooding, many of the physiological responses induced by such stresses and their cellular targets are similar, with the common motif being cellular dehydration. The unifying feature among abiotic stresses is the generation of reactive oxygen species (ROS). High levels of ROS are generated through the impairment of photosynthetic biochemical reactions, while the rates of physiochemical light harvesting are unaffected and ROS production is acentuated by adjustments to the metabolism of respiratory and photorespiratory pathways. Generated ROS include singlet oxygen, superoxide, hydroxyl radical, and hydrogen peroxide, and their presence can cause the oxidation of lipids, nucleic acids, and proteins resulting in cellular damage [30]. The cellular membrane systems and enzyme catalysts are thought to be the primary targets of oxidative stress damage [31]. Cellular membranes are responsible for regulating the passage of solutes in and out of the cell and the cellular compartments. Adverse environmental stress affects both the fluidity of the lipid bilayer and the actions of integral and peripheral membrane-associated proteins. In addition, abiotic

stresses can alter the conformation of protein structures through the disruption of chemical bonds and allosteric modifications of key regulatory enzymes [32, 33].

Natural selection has developed several solutions to protect cellular structures from the adverse effects of abiotic stress. These include the induction of ROS scavanging systems, polyamines, and heat shock proteins. In an effort to negate the toxic effects of ROS, enzymatic and nonenzymatic antioxidant compounds are produced [30, 31]. Some of these compounds are compartmentalized in specific organelles, while others are active throughout the cell. The major antioxidative enzymes include superoxide dismutase (SOD), catalase, peroxidases, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase, and these systems work in concert with metabolites including ascorbic acid, glutathione, α-tocopherol, and β-carotene [30].

Numerous plant studies have demonstrated a strong correlation between the accumulation of compatible osmolytes and an increase in stress tolerance. These organic compounds possess a low molecular weight, are highly soluble, and are nontoxic at high cellular concentrations [34]. It has been hypothesized that these compatible solutes function to adjust the osmotic potential of the cell, to protect against damage from ROS, to stabilize the structure of macromolecules, and to protect membrane integrity, although direct evidence for these functions awaits confirmation [35]. Proline, sucrose, trehalose, polyols, and glycine-betaine form the major compatible osmolytes identified in plants [36]. However, compatible solute accumulation is species specific where different compounds have been chosen by natural selection to accumulate to high cellular concentrations. *Brassica* species appear to accumulate proline and some simple sugars to high concentrations, but not glycine-betaine [37].

In addition to these metabolites, numerous classes of protein demonstrate a positive correlation between their cellular concentration and abiotic stress tolerance. These include the heat shock proteins [38], late-embryogeneis accumulating (LEA) [39], and the cold-responsive proteins (COR) [40]. Numerous studies have focused on the relationship between stress tolerance and the accumulation of these proteins and have revealed an integrated network of genetic pathways that respond to various abiotic stress stimuli and ultimately result in an acclimated cellular state [41, 42].

51.2.2
Hormonal Responses to Abiotic Stress

The interaction and perception of plant hormones is largely responsible for the coordination of plant growth and development [43]. Among these, the phytohormone ABA has often been referred to as a stress hormone due to the substantial number of physiological effects that stress and ABA exposure have in common [44]. A reduction in transpirational water loss through restriction of stomatal aperture, germination inhibition, and reduced leaf expansion are perhaps among the most recognizable effects of ABA [45]. However, ABA also functions to fine-tune plant growth and development under nonstress

conditions by regulating hydraulic conductivity, embryo development, and seed dormancy [45, 46].

The association between ABA and stomatal drought responses was confirmed through ABA-deficient mutant analysis, where the stomata of plants unable to synthesize ABA remained open during dehydration [47–49]. Although increases in ABA concentration are correlated with stress tolerance, the ratio among the phytohormones is important as ABA acts in concert with other phytohormones to elicit the observed physiological responses [50, 51].

ABA signal perception is beginning to be elucidated and uncovering the mechanism involved is an area of intense research [52, 53]. The control of guard cell turgor pressure and thus stomatal aperture is among the best characterized plant cell signaling pathways and involves Ca^{2+} fluxes, G-protein, and F-box protein signaling events, which regulate the efflux of K^{+} cations, anions, and charged metabolites [45, 54–59]. The physiological effects, synthesis, and catabolism of ABA have been extensively covered in a number of additional reviews [43, 60–63]. Stress-induced changes in ABA concentration are rapid and the signals originate in the root before being transmitted systemically throughout the plant. Severe dehydration causes the additional synthesis of ABA in leaf tissues, dramatically increasing ABA concentration resulting in further guard cell turgor pressure changes, adjustments to plant metabolism, and gene expression [64–67]. Ultimately, these genetic and metabolic adjustments allow the plant to acclimate to abiotic stress providing it with enhanced stress tolerance [68, 69]. The profound control that phytohormones hold over plant metabolism [70] and their proximity to stress perception make modification of ABA biochemistry an attractive if daunting target with which to modify stress responses. An improved understanding of ABA physiology and its coordination with additional phytohormones could lead to targeted strategies for improved stress tolerance in plants.

51.2.3
Brassica Stress-Responsive Genes and their Regulatory Pathways

Plants being sessile organisms must respond rapidly to environmental challenges that involve the perception and relay of the stress signal through a multitude of integrated signaling pathways, which ultimately results in changes in the cellular physiology [71, 72]. The mechanisms by which plants perceive the abiotic stress remain unknown although it has been speculated that changes in membrane fluidity, redox potential, cytoskeletal rearrangements, and turgor pressure may each play a role in triggering stress signaling cascades [73–76]. Phytohormones and secondary messengers, including Ca^{2+}, inositol phosphates, and ROS, transmit the primary signal often through multiple phosphorylation cascades, which target regulatory proteins that control stress-related gene expression (Figure 51.3).

The majority of research studing stress signaling in the Brassicas has focused on response to low temperature. The expression levels of numerous genes encoding LEA/COR proteins are induced in response to low-temperature exposure in the Brassicaceae model *A. thaliana* [77] facilitating their identification. Similarly, *Brassica*

transcripts encoding proteins orthologous to the *Arabidopsis* COR proteins accumulate rapidly in response to stress. This is highlighted by the stress response of Bn115, Bn26, and Bn19 from *B. napus*, which are homologous to the *Arabidopsis* COR15a and COR15b proteins [78]. Similarly, *Brassica* homologues of the *Arabidopsis* dehydrins *COR47* and *ERD10* and the low-temperature-inducible *Arabidopsis COR6.6* gene demonstrate analagous expression patterns in response to stress. The low-temperature response of COR/LEA proteins conserved between these species appears to be the first line of cellular defense against the effects of freezing and dehydration in the Brassicaceae with the strongest response observed for COR15. The effect of *Arabidopsis* COR15 on freezing tolerance has been assessed *in planta* [79] and *in vitro* assays [80–82], but no functional analyses of *Brassica* COR15 orthologues have been reported. However, both the solubility of recalcitrant proteins and their protection from denaturation in response to temperature stress were enhanced by the addition of the recombinant *Brassica* LEA proteins BnECP63 and Bn115 [83] suggesting that the function of these *Brassica* homologues and their *Arabidopsis* counterpart COR15 is conserved. Genetic manipulation to induce a suite of appropriate LEA proteins could hold promise for increasing the freezing and dehydrative stress tolerance in *Brassica* species.

Analysis of the promoter region of the *Arabidopsis COR* genes led to the identification of regulatory *cis*-elements named DRE/CRT (drought-responsive elements/C-repeats) [84] and subsequently the transcription factor DREB/CBF (DRE binding factor/C-repeat binding factor) that controlled the low-temperature expression of the *COR* genes [85, 86]. The core sequence (CCGAC) from the DRE/CRT *cis*-element of the *Arabidopsis COR* genes is also found in the promoter sequences of the *Brassica COR* genes and have been demonstrated to be necessary for their low-temperature response [87, 88]. The sequence similarity between the promoters of *Brassica* and *Arabidopsis COR* genes is further conserved with regulatory elements enabling their response to abscisic acid (ABA) found among these orthologues [80–91].

The *DREB1/CBF* transcription factors form a small gene family belonging to the plant-specific AP2 family. They are located as three tightly linked genes on chromosome four of the *Arabidopsis* genome and there is a high degree of conservation between the members of the *DREB1/CBF* family. Furthermore, each member of the *DREB1/CBF* gene family has been demonstrated to induce *COR* gene expression [92, 93]. The *Brassica* orthologues (*BnDREB1/CBF*) are highly homologous to their *Arabidopsis* counterparts [94, 96]. The use of protein sequence alignments of four *B. napus BnDREB1/CBF* transcripts suggested that they separate into two distinct classes represented by *BnCBF17* and *BnCBF16*, *5*, and *7* [95]. This distinction was further highlighted by the differences in binding affinity and specificity observed between BnCBF17 and BnCBF5 [95]. Examination of the expression profiles of *B. napus* DREBs generated in response to low temperature suggested differences between these two classes with induction of one class proceeding those from the second and while the members from both groups were able to bind to DRE/CRT *cis*-elements, only the class I was able to activate downstream gene expression in yeast assays [96]. These observations led to the suggestion that both temporal expression differences and competitive binding between DREB transcription factors played a

role in controlling *COR* gene expression in *B. napus* [96]. However, these functional differences have not been demonstrated *in planta* and analogous studies using class II *DREB* genes from *B. juncea* and *B. napus* indicated they could functionally activate downstream genes in assays involving both yeast and plants [97, 98]. The disagreement on the functionality of class II BnDREBs among these studies may stem from the systems in which the assays were performed (yeast versus *in planta*) and/or difference in expression level of the *BnDREB* constructs.

51.3 Enhancing Environmental Stress Tolerance

Although the physiological responses to different abiotic stresses are related, their duration and the developmental stage most affected by each stress may differ. When grown on saline soils, the plant must tolerate reduced water potential and the presence of toxic ions throughout its life cycle. While drought can occur at any stage throughout a plant's life cycle with the risk of drought increasing over the growing season, for *Brassica* crops anthesis is the most susceptible stage. The risk from freezing stress occurs at the limits of the growing season affecting seedling development and embryo maturity. These differences necessitate stress improvement strategies targeted at particular growth stages to achieve the desired yield protection.

Improvement of crop stress tolerance can by achieved through the direct insertion of small number(s) of carefully selected alleles. A number of promising strategies have been employed to engineer stress-tolerant genotypes in crop plants, and although this technology remains in its infancy, the potential of these improvements is clear. However, for these approaches to be deemed successful, the stress-tolerant germplasm must perform as well as elite lines when grown under normal non-stressed growth conditions and be able to outperform elite lines when grown in a stressful environment, which further complicates the final objective.

51.3.1 Targeted Strategies for Abiotic Stress Improvement in Brassicas

51.3.1.1 Improvement of Freezing Tolerance in Brassicas

The overexpression of *AtDREB1b/CBF1* or *AtDREB1a/CBF3* in transgenic *Arabidopsis* conferred constitutive expression of the *COR* genes, along with a constitutive and enhanced freezing tolerance phenotype [85, 99]. These observations inspired the development of *B. napus* lines overexpressing copies of the *BnDREB* genes. Similar to the results for *Arabidopsis*, overexpression of a class I *BnDREB* gene conferred a significant increase in the level of freezing tolerance, beyond that observed for wild-type controls under nonacclimating conditions, whereas lines overexpressing a class II type demonstrated a smaller increase in freezing tolerance [97]. The importance of the *COR* genes in *Brassica* species was further demonstrated in this study since the difference in freezing tolerance between the transgenic lines was correlated with the

accumulation of the *COR* genes. Preparation to withstand the onset of freezing winter temperatures involves the restructuring of metabolism during a period of acclimation to low but nonfreezing temperatures, and maintenance of photosynthetic capacity is crucial to sustain growth rates to ensure winter survival. The transgenic *BnDREB B. napus* plants constitutively expressing the class I transcription factor presented thickened dark green leaves in addition to the enhanced freezing tolerance, which was accompanied by an increase in photosynthetic capacity and efficiency normally observed in cold-acclimated tissues [97]. Similar adjustments to photosynthetic capacity have been observed in other studies investigating the overexpression of *AtDREB/CBF1* in potato [100] and *AtDREB1a/CBF3* in chrysanthemum [101].

Increases in the level of freezing tolerance can be engineered in *Brassica* species through the constitutive expression of *DREB1/CBF* alleles, although questions remain as to how this can be practically achieved since the increased freezing tolerance was associated with pleiotropic effects including late flowering and dwarfism [97]. The use of such gain-of-function alleles has to occur in combination with tightly regulated promoter sequences that are triggered by specific environmental cues to limit the negative agronomic performance associated with the pleiotropic effects of the gene expression under non-stressed growth conditions [99].

51.3.1.2 Improvement of Drought Tolerance

The water status of a plant depends on balancing the rate of water uptake and the rate of transpiration and it has been estimated that dicotyledonous plant are able to move 90% of the available soil water through transpiration [102]. The rate of water loss from leaves is a function of the size of stomatal aperture, which is highly regulated and under the control of the phytohormone ABA that responds to changes in osmotic homeostasis. A number of genes have been identified that are involved in the synthesis (*era1*) and perception (*abi1, abi2*, and *abi3*) of ABA [46, 103–105]. Attention has focused on ERA1 and ABI1 as targets for the manipulation of stress tolerance as *era1* and *abi1* alleles result in guard cell phenotypes with a greater sensitivity to ABA [54, 105, 106]. Characterization of the *era1* gene led to its identification as a farnesyltransferase, a member of the prenyltransferase proteins. Farnesyltransferases exist as heterodimers consisting of α- and β-subunits that function to modify protein targets possessing the CaaX amino acid motif with the addition of a farnesyl group [107]. *ERA1* encodes for the ~46 kDa β-subunit of farnesyltransferase (FTB), while the ~48 kDa α-subunit is encoded by FTA. In *Arabidopsis*, the deletion of either FTB or inhibition of its farnesylation capacity induces stomatal closure over a wider range of ABA concentrations than ordinarily observed [54, 108]. This altered response reduces water loss at higher soil water potentials and is an attractive target for manipulation to engineer improved stress resistance. The *Arabidopsis era1* mutant possesses a number of pleiotropic effects including delayed growth and development, increased floral organ numbers, and enlarged meristems impeding the realization of its full potential [109, 110]. A number of independent lines were developed by transforming *Arabidopsis* with an *anti-FTA/B* construct under the control of the 35S CaMV constitutive promoter [111]. Germination assays in the

presence of ABA demonstrated that the knock-down alleles possessed intermediate phenotypes compared to wild-type and the *era1* genotypes. The *anti-FTA/B* lines were able to maintain greater hydration levels than the wild-type throughout the period of dehydration and other than showing a short delay in flowering time the *anti-FTA/B* lines resembled a wild-type phenotype [111]. The use of a novel promoter to control the timing of knockdown AtFTA/B alleles has the potential to break the remaining linkage with the pleiotropic effects.

Transferring the phenotype into *Brassica* species is complicated by the additional genome duplication and the lack of a highly efficient transformation strategy. However, a knock-down phenotype for FTB was developed in canola using an *anti-FTB* construct under the control of the *RD29a* promoter [111]. The level of stomatal conductance was assessed in the transgenic lines using gas exchange analyses in both ideal and dehydrated environments. These data indicated that there was no difference in photosynthesis and transpiration rates between control and transgenic plants under adequate water conditions. However, when the plants were allowed to dehydrate, the transgenic lines exhibited an additional 10% reduction in stomatal conductance beyond that observed among control plants [111]. Similar protection from hydration stress was observed when the stress occurred at anthesis where the *anti-FTB* transgenic lines recovered from dehydration faster than their control plants resulting in a reduction in the number of aborted embryos [111].

The promising effects on drought tolerance seen for the *anti-FTB* transgenic canola lines warranted their assessment in randomized field trials. The data from these field experiments replicated those observed under controlled environmental conditions where it was demonstrated that there was no significant difference in yield between the *anti-FTB* lines and the control plants when adequate water was provided. When water was restricted, the transgenic lines were able to produce an additional 10–15% yield beyond that observed in control plants [111]. Further refinement of this technology to reduce the potential for negative pleiotropic effects employed the hydroxypyruvate reductase (*HPR*) gene promoter [102]. HPR is an enzyme that catalyzes the conversion of hydroxypyruvate to glycerate as part of the photorespiratory cycle and the expression of this gene is limited to photosynthetic tissue and is low under ideal conditions but is rapidly induced upon exposure to abiotic stress or high light intensity [102]. Similar levels of yield protection were observed using the *HPR* and *RD29a* promoter sequences in response to hydration [102].

Additional strategies designed to improve drought tolerance originate from genomics studies using canola. *Arabidopsis* microarrays were used to assay heterologous gene expression among imbibed canola seeds and seeds treated with ABA or osmotic stress. It was observed that the expression of 40 genes was induced among these treatments when compared to control seedlings [112]. One of these genes encoded a protein with homology to 11-β-hydroxysteriod dehydrogenase (HSD), an enzyme involved in animal steroid synthesis [113]. Transgenic *Arabidopsis* plants expressing an *Arabidopsis* homologue of the *HSD* gene under the control of the *CaMV 35S* promoter were 20% larger than wild-type controls, with an increase in branch and silique number and in stem diameter. In addition, the transgenic *Arabidopsis* plants tolerated high-salinity concentrations better than their wild-type controls. Similar phenotypic

observations were made when this construct was introduced into canola plants offering great promise as a strategy to modify stress tolerance in *Brassica* [114].

51.3.1.3 Improvement of Salt Tolerance

The vast majority of crop plants including the Brassicas are considered glycophytes, and when grown in saline soils they are subject to both dehydration and ionic stresses. Soil salinity rarely fluctuates and in the majority of cases crops exposed to saline soils will have to endure these stresses throughout their entire life cycle. To tolerate a moderate Na^+ stress it is critical for the plant to maintain a low cytosolic sodium ion concentration. This can be achieved by excluding entry of the ions into the cytosol and removal, sequestration, or secretion of Na^+ ions able to gain entry into the cytosol. Sodium ions are able to gain entry into the cytosol via the potassium ion transporters and can be removed from the cell or sequestered into vacuoles through the action of Na^+/H^+ antiporters. Evidence for the existence of plant antiporters was established through the complementation of Na^+/H^+ antiport mutations in fungi and the characterization of the *SOS1* mutant that identified a plasma membrane antiporter [115, 116].

The difficulties associated with manipulating stress tolerance phenotypes are highlighted in studies overexpressing Na^+ antiporter proteins. The *Arabidopsis* Na^+/H^+ vacuolar antiporter (*AtNHX1*) was isolated by exploiting the homology it shares with its fungal orthologue and its function was confirmed through the rescue of a yeast strain deficient in Na^+ exclusion [117]. The potential of manipulating salt tolerance in plants through the overexpression of the AtNHX1 protein was demonstrated in *Arabidopsis* where transgenic lines exhibiting increased antiporter activity were able to grow under a Na^+ concentration (200 mM) toxic to their wild-type counterparts [115]. These results were replicated in crop species with the introduction of the *AtNHX1* gene into both canola and tomato [118]. The transgenic canola lines overexpressing the *AtNHX1* (*35S::NHX1*) gene produced seed yields comparable to that of the wild-type under control conditions and exceeded wild-type yields exposed to 200 mM NaCl [119]. When these lines were grown under high sodium ion concentrations, the *35S::NHX1* lines accumulated NaCl up to 6% of the dry matter, as the increased antiporter activity sequestered these ions in vacuoles. In these studies, the transgenic plants were found to be substantially equivalent to the wild-type, with minimal changes in fatty acid composition and lipid content of the harvested oil [119].

However, in a recent study assaying the potential additive effects of combining a number of genes proposed to confer salt tolerance, the result observed by Apse *et al.* [115] could not be replicated [120]. Furthermore, this new study demonstrated that there were no improvements observed by combining additional salt tolerance alleles compared to overexpression of an antiporter alone [120]. The discrepancies between these studies might result from the use of different methodologies for measuring salt tolerance. Additional strategies to improve salinity tolerance have involved proteins with less well-defined functions. The ability to improve salinity tolerance through the overexpression of LEA proteins was demonstrated in *Arabidopsis* using the *DNH-5* gene from wheat [121]. This followed similar work using the barley *HVA1* gene whose overexpression was able to improve the salt tolerance of rice

protoplasts [122]. In both these instances, the improvements in salinity tolerance were accompanied by enhanced drought resistance, although the mechanisms causing these phenomena await determination [121, 122].

51.3.2 Nonspecific Approaches to Enhance Abiotic Stress Tolerance

51.3.2.1 Overproduction of Compatible Osmolytes

The correlation of compatible osmolyte accumulation in tissues exhibiting an increased level of stress tolerance was among the first observations made in comparisons between stressed and non-stressed plants. These observations have led to crop improvement strategies focusing on their overproduction to improve stresses including freezing, drought, and excess salinity, where dehydration is a common feature. Compatible solutes can accumulate to high concentrations within the cell without toxic effects and there are many solutes able to act in such a way.

The quaternary ammonium ion glycine-betaine is induced by many plant species under stress and can accumulate in the cell through either metabolic processes or exogenous application [123]. The exogenous application of glycine-betaine to leaves of *B. rapa* resulted in its translocation throughout the entire plant within 24 h [124]. Accumulation of glycine-betaine has been correlated with a stress-tolerant phenotype able to protect against exposure to temperature extremes and an increased salt concentration [125–127]. However, *Brassica* species lack the metabolic pathways necessary to accumulate glycine-betine to high concentrations [128]. Two metabolic pathways have been identified that lead to the synthesis of glycine-betaine that proceeds either through the oxidation of choline or through the methylation of glycine, and the genes encoding the enzymes catalyzing these reactions have been identified and cloned [129–132]. Strategies for stress improvement in *A. thaliana*, *B. napus*, and *B. juncea* have been designed using the overexpression of the choline oxidase gene (*CodA*) to increase the concentration of glycine-betaine in these species [131, 133–135]. Transgenic *B. napus* plants generated in these studies accumulated glycine-betaine to concentrations around 1 μmol g^{-1} FW (fresh weight) and were used in stress tolerance assays examining their response to drought, freezing tolerance, and excess salinity [136]. The transgenic material exhibited greater stress tolerance than their control counterparts leading to the conclusion that increasing the concentration of glycine-betaine was a viable strategy for enhancing stress tolerance in non-accumulating species. However, similar studies also using the *CodA* gene indicated that the glycine-betaine levels were limited by choline availability such that the resultant concentration of glycine-betaine achieved was physiologically insignificant [136]. The subcellular location of glycine-betaine may be an important factor in the efficacy of using this compatible solute since stress tolerance was observed when glycine-betaine accumulated in the chloroplasts [136]. This may explain some of the variation in stress tolerance observed in these studies and suggests that glycine-betaine acts to primarily protect the photosynthetic machinery and thylakoid membranes [135, 137, 138].

Exposure to abiotic stress leads to the accumulation of amino acids in many plant species and proline is by far the most prominent amino acid to accumulate in the *Brassica* species, with higher levels of proline observed in stress-tolerant than stress-sensitive plants [139]. It has been proposed that the accumulation of proline acts in a similar manner to glycine-betaine and functions as a cryo-protectant, a store of nitrogen, an antioxidant, and a compatible osmolyte [140, 141]. Proline is synthesized from glutamine by pyrroline-5-carboxylate synthase (P5CS) and pyyroline-5-caroxylate reductase (P5CR) or from ornithine by ornithine δ-aminotransferase, with glutamate being the major substrate under stressful conditions. The manipulation of cellular proline concentration has been hypothesized to result in an increase in stress tolerance and the efficacy of this was first demonstrated in tobacco where overexpression of *P5CS* resulted in an 18-fold increase in proline concentration and was correlated with increased tolerance to dehydration [142]. However, high proline concentrations are not always associated with an increase in stress tolerance [143]; differences in the methodologies for assessing the stress may once again explain these conflicting observations, but these data suggest that our understanding of proline accumulation is incomplete and this impedes strategies targeting its simple manipulation.

51.3.2.2 **Oxidative Stress Tolerance**

Abiotic stress causes an alteration in metabolic homeostasis and results in an increase in the production of reactive oxygen species particularly in the chloroplast. Accumulation of ROS represents a potent hazard to the cell and pathways have evolved to rapidly metabolize ROS [74, 144], limiting the damage they cause. In addition, at lower concentrations the accumulation of particular ROS (H_2O_2) has been proposed as part of the stress signal transduction network [144, 145]. The manipulation of genes that function in the metabolism of ROS offers another promising strategy to improve tolerance to stress in crop plants. Targets utilized for this strategy have included glutathione S-transferase (GST) [144, 146], peroxidase, super oxide dismutase (SOD) [144, 147–149], asorbate peroxidase, glutathione reductase, or spermine synthase [144, 150].

Improvements in oxidative stress resistance were observed in cold-treated tobacco cells that expressed a pea Cu^{2+}/Zn^{2+} SOD in the chloroplast. It was determined that the transgenic tobacco cells were able to maintain higher levels of photosynthesis than equivalent control cells when subjected to low temperatures [151]. Similar results were obtained in transgenic tobacco lines overexpressing GST or glutathione peroxidase constructs [146]. Further improvements in salinity tolerance were reported by coexpressing catalase with SOD in *B. rapa* suggesting that these effects are additive [152].

51.3.2.3 **Manipulation of Stress Signaling**

In addition to functioning as selective cellular barriers, the phospholipid membranes act as a substrate for the generation of secondary messengers in cell signaling. Phospholipase C (PLC) catalyzes the conversion of phosphotidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Although

not specific to abiotic stress, transient increases in IP_3 concentrations have been associated with exposure to these stimuli [153]. IP_3 is proposed to function as a cellular messenger causing the release of Ca^{2+} ions from internal cellular stores [55], and given this role cellular IP_3 levels must be tightly regulated, which is achieved through the action of inositol polyphosphate kinases and phosphatases. The application of strategies designed to manipulate intercellular signaling has been attempted using a homologue of an inositol polyphosphate kinase obtained from the halophyte *Thellungiella halophila* (*ThIPK2*) [154] and a *B. napus* homologue of *PLC* [155]. Subsequent to promising results observed from initial stress assays in yeast, the consituative expression of *ThIPK2* in transgenic *B. napus* lines resulted in phenotypes with increased tolerance to salinity, drought, and oxidative stress damage [154]. Furthermore, analysis of well-characterized genes involved in abiotic stress tolerance revealed an induction in their expression within the transgenic plants. The overexpression of *PLC* in *B. napus* led to increased photosynthetic capacity and a reduction in stomatal conductance, which indicates a propensity for improved drought tolerance. These results suggest the involvement of inositol polyphosphate kinase in multiple signaling pathways affecting the stress response of *Brassica* species and that engineering of improved stress tolerance can be achieved by direct manipulation of these signaling mechanisms.

51.4
Prospects for Genetic Improvement of Abiotic Stress Tolerance

Abiotic stress tolerance phenotypes are under highly complex genetic and physiological control. This has been highlighted at the molecular level by the interconnected network of interactions that induce the *COR/LEA* gene pathways [156, 157]. The use of molecular techniques and the application of genomics technologies developed for model species have revealed numerous candidate genes for stress improvement [158–160] and have resulted in a number of strategies using single genes to manipulate tolerance to particular stresses or a range of abiotic stresses.

Alternative approaches for stress improvement involve the manipulation of entire plant genomes to combine specific favorable genotypes through recombination. These strategies have accompanied human evolution over the past 10 000 years, achieved initially through fortuitous selection and more recently through targeted breeding programs. The efficiency of these programs has been enhanced by the advent of molecular genetics with the development of high-quality linkage maps that can be used to resolve complex traits by mapping the underlying quantitative trait loci (QTL) that contribute to their variation. This type of analysis is increasing in resolution with the generation of high-density single-nucleotide polymorphism (SNP) arrays [161] that will allow traits with lower heritability to be dissected. These strategies are now being combined with the analysis of genome-wide transcription profiles to identify expression QTL (eQTL) associated with traits of interest [162] that might be incorporated in breeding strategies. The high-density marker and expression platforms also offer the promise of genome-wide association (GWA)

studies that have been successfully exploited in the study of human disease [163]. However, the application of genomics technologies in plant breeding is still in its infancy and while GWA studies are becoming feasible in crops [164], the practical application of such methods will not be realized for sometime.

51.4.1 Traditional Breeding and QTL Mapping

The selection of key agronomic traits over the thousands of years of crop domestication made dramatic advances in crop improvement. The philosophy behind the Green Revolution was to provide optimal growth environments for elite genotypes that are able to outperform and ultimately replace local landraces [2, 165, 166]. The major breeding objectives for elite genotype development of the *Brassica*s have been directed toward yield improvement and hybrid production, quality enhancement, [167, 168], pod-shattering and lodging resistance [169–172], early maturation, and disease resistance [173–175], with little resources targeted at environmental stress tolerance.

Tolerance to abiotic stress is controlled by genes at multiple loci, which often possess pleiotropic interactions with loci controlling yield characteristics, which complicates breeding strategies. These difficulties are compounded as selection for stress tolerance under field conditions is problematic due to the inability to consistently apply a specific stress and adequately monitor subtle quantitative phenotypes. Furthermore, since the underlying physiological mechanisms controlling stress tolerance are poorly understood, pertinent selections for material with increased stress tolerance is challenging [176–178]. These problems can be circumvented through the use of physiological assays made in artificial laboratory environments to direct selection. Numerous such assays have been utilized to monitor stress tolerance including ion leakage [179, 180], tissue water content [181], meristem regrowth after freezing [182], ABA concentration [183], and chloroplast function [184, 185]. Reproducible data generated from these assays facilitate reliable quantitative assessments of stress tolerance and the dissection of these complex traits. The association of a few loci with large effects with robust molecular markers will enhance crop improvement and allow the manipulation of complex traits without the requirement for specific controlled environmental conditions for phenotypic assessment. The cost and time required for conventional breeding programs can be reduced considerably through the adoption of such approaches as has been demonstrated by the improvement of drought tolerance in maize [186].

The application of molecular genetic technologies has identified the linkage groups of the six *Brassica* species confirming the cytogenetic relationships first described in the triangle of U [5, 186–194] along with those to more distantly related species [195]. The density of the molecular markers describing these linkage groups allows the dissection of complex traits such as abiotic stress tolerance into individual QTL facilitating the identification of the underlying chromosomal regions controlling stress tolerance. Largely QTL mapping in Brassicas has focused on easily

measured traits; however, a large number of QTL, many with small effects, were identified in *B. rapa* and *B. napus*, highlighting the polygenic nature, and revealing the complex genetic interactions contributing to winter survival, vernalization, and freezing tolerance in these species [26, 196, 197]. However, these QTL were responsible for a small fraction of variability identified within each population compromising their use in a marker-assisted breeding strategy.

51.4.2 Promise of Genomics Technologies to Elucidate Stress Tolerance

The *Brassica* species and the model plant *A. thaliana* share a close phylogenetic relationship, which allows the results from research focused on stress physiology in *Arabidopsis* to be directly exploited in *Brassica* crop improvement. Genomics strategies have been extensively applied in *Arabidopsis* to study abiotic stress tolerance since such complex traits with their multifaceted plant response can benefit from the application of robust but wide-ranging analyses. Such analyses have been gaining traction in other crops and research in *Brassica* species is beginning to take advantage of the new opportunities that are likely to gain in momentum over the next few years.

51.4.2.1 Genetic Screens to Elucidate Plant Stress Responses

Forward genetic screening of plant populations enriched with mutations induced through the use of chemical, physical, or genetic perturbations can not only identify improved phenotypes but can also facilitate gene discovery for loci controlling stress responses. Such screening strategies have been applied successfully to identify *Arabidopsis* lines with enhanced [198, 199] or reduced [200, 201] freezing tolerance. Furthermore, the use of genetic screens assaying for changes in the activity of a reporter gene under the control of the *RD29a* promoter revealed the complex network of interactions underlying the expression of the *COR/ERD/RD* genes [202]. The expression of *RD29a* is induced by dehydration, ABA, salinity, and low-temperature exposure. Analysis of the mutants obtained through the *RD29a* reporter gene screen revealed salt overly sensitive (SOS), constitutive (COS), high (HOS) and low (LOS) expression of osmotic response mutants [32, 203–205]. The identification and characterization of these mutants has enabled the genetic dissection of many of the components involved in stress signal transduction [206–208]. The success of these screens has led to a similar strategy being employed focusing on HSP70, a heat shock protein involved in the unfolded protein response. This screen has identified a potential sensor for temperature as a protein component of the nucleosome [209]. The increasing knowledge gained from studies conducted in model organisms has and will continue to provide additional targets for improvement in *Brassica* species and allow for the refinement of current strategies [210].

51.4.2.2 Global Transcriptome Analyses

The application of high-throughput genomics technologies to stress gene discovery has led to the promise of easy gene identification. The use of differential cDNA

expression [77, 211], microarray [212–216], and serial analysis of gene expression [159, 217] has revealed sets of differentially expressed stress-responsive genes. The majority of exploratory genomics research utilizes resources developed for *Arabidopsis* due to their availability and comparatively low cost, but the development of high-throughput technology platforms designed specifically for *Brassica* species will allow more comprehensive assessments of *Brassica* transcriptomes [215, 216, 218]. These technologies have the potential to identify pathways and candidate genes for stress tolerance that can be used in similar targeted strategies as those described in Section 51.3.

51.4.2.3 The Allure of Whole-Genome Sequences

Sequencing of the *Arabidopsis* genome has identified ~30 000 genes and determining their function is an ongoing enterprise [6]. At present, there are 1876 genes annotated as being involved in abiotic stress, 1778 genes in transport, and 724 genes in intracellular signaling. Multiple rounds of whole-genome duplication have occurred throughout the natural history of the Brassicaceae and it has been proposed that the genomes of the diploid *Brassica* were triplicated relative to *Arabidopsis* [219, 220]. The resulting genome complexity present among the *Brassica* species provides additional material for evolutionary adaptations and might explain why these species exhibit an increased capacity to withstand stressful environments beyond that of *Arabidopsis*. There are projects that have been launched to assemble the genome sequences for all the *Brassica* diploids [221] (http://canseq.ca). Such studies have already uncovered significant expansion of gene families related to hormone biosynthesis within the *B. rapa* genome compared to *Arabidopsis* [222], which suggest that understanding the impact of genome evolution within the *Brassica* species might provide insight into the phenotypic variance observed within this genus.

The availability of *Brassica* genome sequences will quickly facilitate genetic studies with the development of dense genome-wide marker sets. Genome-wide association (GWA) is a powerful technique that unlike QTL mapping does not require the development of large segregating mapping populations, but utilizes the ancient recombination events that are inherited within diverse collections to resolve loci controlling observable phenotypes. Association mapping requires the ability to query genome-wide SNPs to uncover those closely associated through linkage disequilibrium with QTL and has great potential to become a gene-tagging tool in crop plants [164]. The power of this approach has been demonstrated in the crucifer *Arabidopsis* for flowering time [223] and pathogen resistance [224]. Although this technology awaits exploitation in Brassicas, it has been successfully applied to other crop species [225]. With the development of fixed foundation diversity sets (www.brassica.info) for different species and the characterization of large SNP collections [226], the tools to realize this powerful strategy in Brassicas will be available.

The availability of whole-genome sequences for the *Brassica* species will bring together linkage analysis, positional cloning, and gene expression data that will reveal the link between genes and the phenotypes they control. Transcript levels when measured in defined mapping populations can be considered as quantitative traits

and the variation observed among the progeny used to map eQTL [227]. The transcript level is determined by many factors; in some instances, the eQTL can be used as a marker for a particular phenotype and the gene underlying the eQTL might provide a good candidate for a phenotypic QTL. Expression of QTL can be classified as either *cis* or *trans*, with eQTL colocated at their target gene thought to be *cis*-acting, whereas eQTL found to be unlinked to their target gene inferred to have a *trans*-acting function. These latter eQTL have the potential to resolve major regulators of gene expression where an eQTL has an effect on the expression of groups of genes. Global eQTL mapping has been applied in *Arabidopsis* [228] and more recently in crops such as maize and barley [229]. For the interpretation of complex traits such as abiotic stress tolerance, the ability to apply these types of systems-based analyses is probably the most promising [230].

51.4.2.4 The Added Complexity that Epigenetic Changes Impact on Abiotic Stress Tolerance

Genomic DNA is organized into chromatin through the action of histone proteins with the basic unit being the nucleosome. Chromatin structure is dynamic and is modified during various physiological processes by specific and highly regulated epigenetic factors that adjust the affinity of histone–DNA interactions. Epigenetic factors increase the diversity and complexity present among organisms, and within an organism, as a single embryonic genome gives rise to a multitude of epigenomes that result in differential cell fate and tissue differentiation. The processes underlying these epigenetic changes include DNA methylation, histone modifications (e.g., methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation), and the action of small RNAs and transposon elements, which affect the expression rather than the transmission of particular alleles.

The action of small RNAs results in post-transcriptional gene silencing in complementary target mRNAs. There have been several small RNAs identified from *Arabidopsis* that are implicated in abiotic stress regulation. These include elements complementary to *AAO3*, a drought-responsive enzyme involved in ABA biosynthesis, a lypoxygenase, and small RNAs created by the 3′end overlap between *SRO5* and pyrroline-5-carboxylate dehydrogenase (*P5CDH*) [231]. *P5CDH* is a single gene that is ubiquitously expressed and is involved in proline catabolism and *SRO5* is responsive to salt stress [231, 232]. It has been demonstrated that in response to salt stress the complimentary base pairing between *SRO5* and *P5CDH* transcripts activates the siRNA pathway resulting in a reduction of *P5CDH* transcript accumulation and a subsequent increase in proline accumulation [231, 232]. Such epigenetic mechanisms add an additional layer of complexity to stress-inducible gene regulation, and with the identification 30 families of miRNA and siRNA with many possessing stress-dependant expression, it is likely this phenomenon forms an integral part of abiotic stress gene regulation and that it affects a wide range of gene functions [231].

In plants, cytosine methylation of DNA occurs both at the symmetrical CpG and CpNpG loci and at the asymmetrical CpHpH loci; the symmetrical patterns are transmitted through meiosis, whereas asymmetrical patterns must be reestablished

at each generation. Genome-wide methylation patterns in *Arabidopsis* revealed that the heterochromatin rich in repetitive and transposable elements in pericentric chromosomal regions were heavily methylated. Genes possessing methylated promoter regions were more likely to be expressed in tissue-specific fashion, whereas genes methylated along coding regions were constitutively expressed [233]. Evidence indicates that abiotic stresses including low temperature and salinity cause hypomethylation of targeted loci which coincides with increased gene expression in these hypomethylated regions.

The components involved and the extent to which epigenetic changes effect gene expression, particularly how a stress memory might be stored, are only now being realized [234]. A more complete understanding of the regulation of these phenomena offers additional techniques with which to manipulate stress tolerance along with perhaps providing strategies for fine-tuning present transgenic approaches [235].

51.5
Alternative Sources of Stress-Tolerant Alleles

The identification of desirable characteristics from genetic variation found within species has led over generations to the development of adapted crop varieties. The continued improvement and expanded use of elite breeding lines has improved yield potential, but ultimately it has effectively limited the available gene pool [236]. The elite cultivars are often grown under ideal environments and are developed under intense selection pressure for improvements in yield, quality, and ease of harvesting [237, 238]. Alleles that provide additional protection against the effects of abiotic stresses often have negative net effects on yield components and have been discarded during the development of such lines. Limited genetic resources among *Brassica* crop species have led to more creative strategies for identifying potential genetic variation, with the development of resynthesized lines [239], induced mutagenized lines [240], somaclonal variants [241], induced epialleles [235], and interspecific hybridizations [242], often achieved only after embryo rescue [243].

However, extensive genetic variation exists among noncultivated *Brassica* species, landraces, and more distantly related species within the Brassicaceae. Assessment of such germplasm offers great potential for crop improvement. Although the prospect of introducing traits into *Brassica* crops from species beyond the *Brassica* genus through sexual reproduction is limited, unless a successful bridging species can be identified, analysis of exotic species will offer valuable insight, into the natural history of stress tolerance and genome evolution. The investigation of stress-tolerant species belonging to the Brassicaceae has begun with the development of EST libraries and T-DNA mutagenized populations of *T. halophila* [244–247]. *Thlaspi arvense*, a plant able to withstand the extremely harsh winters on the Canadian Prairies, has been investigated revealing its genetic architecture in relation to stress tolerance genes and how its transcriptome responds to low temperature [248–250]. It is anticipated that genetic and physiological investigation of these hardy species will reveal novel

genes and alleles that confer stress tolerance that can be captured and employed in *Brassica* crop improvement strategies.

51.6 Conclusions

In the face of a growing and discerning population, the provision of a plentiful affordable food supply requires further increases in agricultural production. This necessitates the expansion of agriculture to marginal lands that will entail the development of crop varieties adapted to harsher environments. All sessile organisms require tolerance to abiotic stress; however, the improvement of abiotic stress tolerance has received little attention in modern crop development, which has primarily focused on harvest index, quality parameters, and disease resistance. Tolerance to abiotic stress must become a priority in breeding programs, utilizing both traditional and transgenic approaches. The use of transgenic strategies for crop improvement holds great promise; however, to be considered successful, the genetic manipulation must provide enhanced yields under stressful environments while not negatively affecting yield under ideal environments. Seed yield is a complex trait, which is affected by a large number of factors throughout plant growth and development. The induction of enhanced stress tolerance must be targeted to specific growth stages, particular organs, or environmental cues. This requires the identification and characterization of highly regulated and specific promoter sequences to be ultimately successful. Owing to the genetic complexities of abiotic stress tolerance and the interaction with yield factors, it is unlikely that a single strategy will offer a panacea for abiotic stress in *Brassica* species. Fortunately, the rapid development of genomics tools for the *Brassica* oilseeds promises avenues for resolving the complexities and harnessing this knowledge to develop superior crop varieties.

References

1 UN (2009) World Population Prospects: The 2008 Revision. Population Newsletter.
2 Khush, G. (1999) Green Revolution: preparing for the 21st century. *Genome*, **42**, 646–655.
3 Campbell, C.J. (1997) *The Coming Oil Crisis*, Multi-Science Publishing.
4 Campbell, C.J. (2005) *Oil Crisis*, Multi-Science Publishing.
5 U. (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japan J. Bot.*, **4**, 389–452.
6 The Arabidopsis Genome Initiative (2000) Analysis of the genome of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796–815.
7 Downey, R.K. (1990) *Brassica* oilseed breeding: achievements and opportunities. *Plant Breed. Abstr.*, **60**, 1165–1170.
8 Downey, R.K. and Bolton, J.L. (1961) *Production of Rapeseed in Western Canada*, Dept. of Agriculture, Ottawa.
9 FAO (2009) *FAO Yearbook*, Food and Agriculture Organisation.
10 Boyer, J.S. (1982) Plant Productivity and Environment. *Science*, **218**, 443–448.

11 White, G.F. and Haas, J.E. (1975) *Assessment of Research on Natural Hazards*, MIT Press, Cambridge, Massachusetts.

12 Levitt, J. (1980) *Responses of Plants to Environmental Stresses: Chilling, Freezing and High Temperature Stresses*, 2nd edn, Academic Press, New York.

13 Good, A.G. and Maclagan, J.L. (1993) Effects of drought stress on the water relations in *Brassica* species. *Can. J. Plant Sci.*, **73**, 525–529.

14 Richards, R.A. and Thurling, N. (1978) Variation between and within species of rapeseed (*Brassica campestris* and *B. napus*) in response to drought stress. II. Sensitivity at different stages of development. *Aust. J. Agric. Res.*, **29**, 479–490.

15 Richards, R.A. and Thurling, N. (1978) Variation between and within species of rapeseed (*Brassica campestris* and *B. napus*) in response to drought stress. I. Sensitivity at different stages of development. *Aust. J. Agric. Res*, **29**, 469–477.

16 Dhawan, A.K. (1985) Freezing in oilseed *Brassica* species: some factors affecting injury. *J. Agric. Sci. (Camb.)*, **104**, 513–518.

17 Johnson-Flanagan, A., Singh, J., and Thiagarajah, M. (1990) The impact of sublethal freezing during maturation of pigment content in seeds of *Brassica napus*. *J. Plant Physiol.*, **136**, 385–390.

18 Morgan, J.M. (1980) Possible role of abscisic acid in reducing seed set in water stressed wheat plants. *Nature*, **285**, 655–657.

19 Lalk, I. and Dörffling, K. (1985) Hardening, abscisic acid, proline and freezing resistance in two winter wheat varieties. *Physiol. Plant.*, **63**, 287–292.

20 Leone, A., Costa, A., Grillo, S., Tucci, M., Horvath, I., and Vigh, L. (1996) Acclimation to low water potential determines changes in membrane fatty acid composition and fluidity in potato cells. *Plant Cell Environ.*, **19**, 1103–1109.

21 Jamaux, I., Steinmetz, A., and Belhassen, E. (1997) Looking for molecular and physiological markers of osmotic adjustment in sunflower. *New Phytol.*, **137**, 117–127.

22 Volkmar, K.M., Hu, H., and Steppuhn, H. (1998) Physiological responses of plants to salinity: a review. *Can. J. Plant Sci.*, **78**, 19–27.

23 Morgan, J.M. (2003) Osmoregulation and water stress in higher plants. *Annu. Rev. Plant Physiol.*, **35**, 299–319.

24 Richards, R.A. (1978) Genetic analysis of drought stress response in rapeseed (*Brassica campestris* and *B. napus*). I. Assessment of environments for maximum selection response in grain yield. *Euphytica*, **27**, 609–615.

25 Francois, L. (1994) Growth, seed yield, and oil content of canola grown under saline conditions. *Agron. J.*, **86**, 233–237.

26 Teutonico, R.A. and Osborn, T.C. (1995) Mapping loci controlling vernalization requirement in *Brassica rapa*. *Theor. Appl. Genet.*, **91**, 1279–1283.

27 Steppuhn, H., Volkmar, K.M., and Miller, P.R. (2001) Comparing canola, field pea, dry bean, and durum wheat crops grown in saline media. *Crop Sci.*, **41**, 1827–1833.

28 Rameeh, V., Rezai, A., and Saeidi, G. (2004) Study of salinity tolerance in rapeseed. *Commun. Soil. Sci. Plan*, **35**, 2849–2866.

29 Ghobadi, M., Bakhshandeh, M., Fathi, G., Gharineh, M.H., Alami-Said, K., Naderi, A., and Ghobadi, M.E. (2006) Short and long periods of water stress during different growth stages of canola (*Brassica napus* L.): effect on yield, yield components, seed oil and protein contents. *J. Agron.*, **5**, 336–341.

30 Bartosz, G. (1997) Oxidative stress in plants. *Acta Physiol. Plant.*, **19**, 47–64.

31 Møller, I.M., Jensen, P.E., and Hansson, A. (2007) Oxidative modifications to cellular components in plants. *Annu. Rev. Plant. Biol.*, **58**, 459–481.

32 Lee, I., Bender, E., and Kadenbach, B. (2002) Control of mitochondrial membrane potential and ROS formation by reversible phosphorylation of cytochrome *c* oxidase. *Mol. Cell Biochem.*, **234–235**, 63–70.

33 Vanlerberghe, G.C., Yip, J.Y.H., and Parsons, H.L. (1999) *In organello* and *in vivo* evidence of the importance of the regulatory sulfhydryl/disulfide system and pyruvate for alternative oxidase

activity in tobacco. *Plant Physiol.*, **121**, 793–803.
34 Yancey, P.H. (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.*, **208**, 2819–2830.
35 Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1997) Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol.*, **38**, 1095–1102.
36 Majumder, A.L., Sengupta, S., and Goswami, L. (2010) Osmolyte Regulation in Abiotic Stress, pp. 349–370.
37 Gibon, Y., Bessieres, M.A., and Larher, F. (1997) Is glycine betaine a non-compatible solute in higher plants that do not accumulate it? *Plant Cell Environ.*, **20**, 329–340.
38 Waters, E.R., Lee, G.J., and Vierling, E. (1996) Evolution, structure and function of the small heat shock proteins in plants. *J. Exp. Bot.*, **47**, 325–338.
39 Goyal, K., Walton, L.J., and Tunnacliffe, A. (2005) LEA proteins prevent protein aggregation due to water stress. *Biochem. J.*, **388**, 151–157.
40 Thomashow, M.F. (1998) Role of cold-responsive genes in plant freezing tolerance. *Plant Physiol.*, **118**, 1–8.
41 Thomashow, M.F. (2001) So what's new in the field of plant cold acclimation? Lots!. *Plant Physiol.*, **125**, 89–93.
42 Thomashow, M.F. (2003) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.*, **50**, 571–599.
43 Davies, P.J. (2010) The Plant Hormones: Their Nature, Occurrence, and Functions, pp. 1–15.
44 Munns, R. and Sharp, R.E. (1993) Involvement of abscisic-acid in controlling plant-growth in soils of low water potential. *Aust. J. Plant Physiol.*, **20**, 425–437.
45 Leung, J. and Giraudat, J. (1998) Abscisic acid signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **49**, 199–222.
46 Finkelstein, R.R., Gampala, S.S.L., and Rock, C.D. (2002) Abscisic acid signaling in seeds and seedlings. *Plant Cell*, **14**, S15–S45.
47 Koornneef, M., Hanhart, C.J., Hilhorst, H.W.M., and Karssen, C.M. (1989) *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. *Plant Physiol.*, **90**, 463–469.
48 Rock, C.D. and Zeevaart, J.A. (1991) The aba mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. *Proc. Natl. Acad. Sci. USA*, **88**, 7496–7499.
49 Léon-Kloosterziel, K.M., Gil, M.A., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A., and Koornneef, M. (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. *Plant J.*, **10**, 655–661.
50 Liu, Y., Ye, N., Liu, R., Chen, M., and Zhang, J. (2010) H_2O_2 mediates the regulation of ABA catabolism and GA biosynthesis in *Arabidopsis* seed dormancy and germination. *J. Exp. Bot.*, **61** (11), 2979–2990.
51 Sally, W. and William, J.D. (2010) Drought, ozone, ABA and ethylene: new insights from cell to plant to community. *Plant Cell Environ.*, **33**, 510–525.
52 Hirayama, T. and Shinozaki, K. (2007) Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends Plant Sci.*, **8**, 343–351.
53 Klingler, J., Batelli, G., and Zhu, J. (2010) ABA receptors: the START of a new paradigm in phytohormone signalling. *J. Exp. Bot.*, **12**, 3199–3210.
54 Pei, Z.-M., Ghassemian, M., Kwak, C.M., McCourt, P., and Schroeder, J.I. (1998) Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. *Science*, **282**, 287–290.
55 Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M., and Waner, D. (2001) Guard cell signal transduction. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.*, **52**, 627–658.
56 Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., and Abrams, S.R. (2010) Abscisic acid: emergence of a core signaling

network. *Annu. Rev. Plant. Biol.*, **61**, 651–679.

57 Kim, T.-H., Böhmer, M., Hu, H., Nishimura, N., and Schroeder, J.I. (2010) Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. *Annu. Rev. Plant. Biol.*, **61**, 561–591.

58 Roelfsema, M.R.G. and Hedrich, R. (2010) Making sense out of Ca^{2+} signals: their role in regulating stomatal movements. *Plant Cell Environ.*, **33**, 305–321.

59 Zhao, Z., Stanley, B.A., Zhang, W., and Assmann, S.M. (2010) ABA-regulated G protein signaling in *Arabidopsis* guard cells: a proteomic perspective. *J. Proteome. Res.*, **9**, 1637–1647.

60 Cutler, A.J. and Krochko, J.E. (1999) Formation and breakdown of ABA. *Trends Plant Sci.*, **4**, 472–478.

61 Seo, M. and Koshiba, T. (2002) Complex regulation of ABA biosynthesis in plants. *Trends Plant Sci.*, **7**, 41–48.

62 Zhou, R., Cutler, A.J., Ambrose, S.J., Galka, M.M., Nelson, K.M., Squires, T.M., Loewen, M.K., Jadhav, A.S., Ross, A.R.S., Taylor, D.C., and Abrams, S.R. (2004) A new abscisic acid catabolic pathway. *Plant Physiol.*, **134**, 361–369.

63 Wasilewska, A., Vlad, F., Sirichandra, C., Redko, Y., Jammes, F., Valon, C., Frey, N.F.d., and Leung, J. (2008) An update on abscisic acid signaling in plants and more. *Mol. Plant.*, **1**, 198–217.

64 Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.*, **2**, 48–54.

65 Christmann, A., Moes, D., Himmelbach, A., Yang, Y., Tang, Y., and Grill, E. (2006) Integration of abscisic acid signalling into plant responses. *Plant Biol. (Stuttg.)*, **8**, 314–325.

66 Alexander, C., Elmar, W.W., Ernst, S., and Erwin, G. (2007) A hydraulic signal in root-to-shoot signalling of water shortage. *Plant J.*, **52**, 167–174.

67 Huang, D., Wu, W., Abrams, S.R., and Cutler, A.J. (2008) The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. *J. Exp. Bot.*, **59**, 2991–3007.

68 Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993) The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of rd22, a gene responsive to dehydration stress in *Arabidopsis thaliana*. *Mol. Gen. Genet.*, **238**, 17–25.

69 Seki, M., Umezawa, T., Urano, K., and Shinozaki, K. (2007) Regulatory metabolic networks in drought stress responses. *Curr. Opin. Plant. Biol.*, **10**, 296–302.

70 Hey, S.J., Byrne, E., and Halford, N.G. (2010) The interface between metabolic and stress signalling. *Ann. Bot.*, **105**, 197–203.

71 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Ann. Rev. Plant Biol.*, **57**, 781–803.

72 Nakashima, K., Ito, Y., and Yamaguchi-Shinozaki, K. (2009) Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. *Plant. Physiol.*, **149**, 88–95.

73 Murata, N. and Los, D.A. (1997) Membrane fluidity and temperature perception. *Plant Physiol.*, **115**, 875–879.

74 Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.*, **55**, 373–399.

75 Drøbak, B.K., Franklin-Tong, V.E., and Staiger, C.J. (2004) The role of the actin cytoskeleton in plant cell signaling. *New Phytologist.*, **163**, 13–30.

76 Nakagami, H., Pitzschke, A., and Hirt, H. (2005) Emerging MAP kinase pathways in plant stress signalling. *Trends Plant Sci.*, **10**, 339–346.

77 Gilmour, S.J., Artus, N.N., and Thomashow, M.F. (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. *Plant Mol. Biol.*, **18**, 13–21.

78 Weretilnyk, E., Orr, W., White, T.C., Iu, B., and Singh, J. (1993) Characterization of three related low-temperature-regulated cDNAs from winter *Brassica napus*. *Plant Physiol.*, **101**, 171–177.

79 Artus, N.N., Uemura, M., Steponkus, P.L., Gilmour, S.J., Lin, C., and Thomashow, M.F. (1996) Constitutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. *Proc. Natl. Acad. Sci. USA*, **93**, 13404–13409.

80 Uemura, M., Gilmour, S.J., Thomashow, M.F., and Steponkus, P.L. (1996) Effects of COR6.6 and COR15am polypeptides encoded by COR (cold-regulated) genes of *Arabidopsis thaliana* on the freeze-induced fusion and leakage of liposomes. *Plant Physiol.*, **111**, 313–327.

81 Webb, M.S., Gilmour, S.J., Thomashow, M.F., and Steponkus, P.L. (1996) Effects of COR6.6 and COR15am polypeptides encoded by COR (cold-regulated) genes of *Arabidopsis thaliana* on dehydration-induced phase transitions of phospholipid membranes. *Plant Physiol.*, **111**, 301–312.

82 Nakayama, K., Okawa, K., Kakizaki, T., Honma, T., Itoh, H., and Inaba, T. (2007) *Arabidopsis* Cor15am is a chloroplast stromal protein that has cryoprotective activity and forms oligomers. *Plant Physiol.*, **144**, 513–523.

83 Singh, J., Whitwill, S., Lacroix, G., Douglas, J., Dubuc, E., Allard, G., Keller, W., and Schernthaner, J.P. (2009) The use of Group 3 LEA proteins as fusion partners in facilitating recombinant expression of recalcitrant proteins in *E. coli*. *Protein Expr. Purif.*, **67**, 15–22.

84 Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA*, **94**, 1035–1040.

85 Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O., and Thomashow, M.F. (1998) *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science*, **280**, 104–106.

86 Shinwari, Z.K., Nakashima, K., Miura, S., Kasuga, M., Seki, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998) An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. *Biochem. Biophys. Res. Commun.*, **250**, 161–170.

87 White, T.C., Simmonds, D., Donaldson, P., and Singh, J. (1994) Regulation of BN115, a low-temperature-responsive gene from winter *Brassica napus*. *Plant Physiol.*, **106**, 917–928.

88 Jiang, C., Iu, B., and Singh, J. (1996) Requirement of a CCGAC *cis*-acting element for cold induction of the BN115 gene from winter *Brassica napus*. *Plant Mol. Biol.*, **30**, 679–684.

89 Yamaguchi-Shinozaki, K. and Shinozaki, K. (1993b) *Arabidopsis* DNA encoding two desiccation-responsive rd29 genes. *Plant Physiol.*, **101**, 1119–1120.

90 Knight, H., Zarka, D.G., Okamoto, H., Thomashow, M.F., and Knight, M.R. (2004) Abscisic acid induces CBF gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. *Plant Physiol.*, **135**, 1710–1717.

91 Yamaguchi-Shinozaki, K. and Shinozaki, K. (2005) Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci.*, **10**, 88–94.

92 Medina, J., Bargues, M., Terol, J., Perez-Alonso, M., and Salinas, J. (1999) The *Arabidopsis* CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. *Plant Physiol.*, **119**, 463–470.

93 Thomashow, M.F., Gilmour, S.J., Stockinger, E.J., Jaglo-Ottosen, K.R., and Zarka, D.G. (2001) Role of the *Arabidopsis* CBF transcriptional activators in cold acclimation. *Physiol. Plant*, **112**, 171–175.

94 Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T., and Thomashow, M.F. (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.*, **127**, 910–917.

95 Gao, M.-J., Allard, G., Byass, L., Flanagan, A.M., and Singh, J. (2002) Regulation and characterization of four CBF transcription factors from *Brassica napus*. *Plant Mol. Biol.*, **49**, 459–471.

96 Zhao, T.-J., Sun, S., Liu, Y., Liu, J.-M., Liu, Q., Yan, Y.-B., and Zhou, H.-M. (2006) Regulating the drought-responsive element (DRE)-mediated signaling pathway by synergic functions of *trans*-active and *trans*-inactive DRE binding factors in *Brassica napus*. *J. Biol. Chem.*, **281**, 10752–10759.

97 Savitch, L.V., Allard, G., Seki, M., Robert, L.S., Tinker, N.A., Huner, N.P.A., Shinozaki, K., and Singh, J. (2005) The effect of overexpression of two *Brassica* CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. *Plant Cell Physiol.*, **46**, 1525–1539.

98 Cong, L., Chai, T., and Zhang, Y. (2008) Characterization of the novel gene BjDREB1B encoding a DRE-binding transcription factor from *Brassica juncea* L. *Biochem. Biophys. Res. Commun.*, **371**, 702–706.

99 Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotech.*, **17**, 287–291.

100 Pino, M., Skinner, J.S., Jeknic, Z., Hayes, P.M., Soeldner, A.H., Thomashow, M.F., and Chen, T.H.H. (2008) Ectopic AtCBF1 over-expression enhances freezing tolerance and induces cold acclimation-associated physiological modifications in potato. *Plant Cell Environ.*, **31**, 393–406.

101 Hong, B., Ma, C., Yang, Y., Wang, T., Yamaguchi-Shinozaki, K., and Gao, J. (2009) Over-expression of AtDREB1A in chrysanthemum enhances tolerance to heat stress. *Plant Mol. Biol.*, **70**, 231–240.

102 Wang, Y., Beaith, M., Chalifoux, M., Ying, J., Uchacz, T., Sarvas, C., Griffiths, R., Kuzma, M., Wan, J., and Huang, Y. (2009) Shoot-specific down-regulation of protein farnesyltransferase ({alpha}-subunit) for yield protection against drought in canola. *Mol. Plant.*, **2**, 191–200.

103 Koornneef, M., Reuling, G., and Karssen, C.M. (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiol. Plant*, **61**, 377–383.

104 Koornneef, M., Hanhart, C.J., Hilhorst, H.W.M., and Karssen, C.M. (1989) *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. *Plant Physiol.*, **90**, 463–469.

105 Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S., and McCourt, P. (1996) A protein farnesyltransferase involved in abscisic acid signal transduction in *Arabidopsis*. *Science*, **273**, 1239–1241.

106 Hugouvieux, V., Kwak, J.M., and Schroeder, J.I. (2001) An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in *Arabidopsis*. *Cell*, **106**, 477–487.

107 Lane, K.T. and Beese, L.S. (2006) Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyl transferase type I. *J. Lipid Res.*, **47**, 681–699.

108 Allen, G.J., Murata, Y., Chu, S.P., Nafisi, M., and Schroeder, J.I. (2002) Hypersensitivity of abscisic acid-induced cytosolic calcium increases in the *Arabidopsis* farnesyltransferase mutant era1-2. *Plant Cell*, **14**, 1649–1662.

109 Donetta, D., Bayliss, P., Sun, S., Sage, T., and McCourt, P. (2000) Farnesylation is involved in meristem organization in *Arabidopsis*. *Planta*, **211**, 182–190.

110 Running, M.P., Lavy, M., Sternberg, H., Galichet, A., Gruissem, W., Hake, S., Ori, N., and Yalovsky, S. (2004) Enlarged meristems and delayed growth in plp mutants result from lack of CaaX prenyltransferases. *Proc. Natl Acad. Sci.*, **101**, 7815–7820.

111 Wang, Y., Ying, J., Kuzma, M., Chalifoux, M., Sample, A., McArthur, C., Uchacz, T., Sarvas, C., Wan, J., Dennis, D.T., McCourt, P., and Huang, Y. (2005) Molecular tailoring of farnesylation for

plant drought tolerance and yield protection. *Plant J.*, **43**, 413–424.

112 Li, F., Wu, X., Tsang, E., and Cutler, A.J. (2005) Transcriptional profiling of imbibed *Brassica napus* seed. *Genomics*, **86**, 718–730.

113 Tomlinson, J.W., Walker, E.A., Bujalska, I.J., Draper, N., Lavery, G.G., Cooper, M.S., Hewison, M., and Stewart, P.M. (2004) 11{beta}-Hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. *Endocr. Rev.*, **25**, 831–866.

114 Li, F., Asami, T., Wu, X., Tsang, E.W.T., and Cutler, A.J. (2007) A putative hydroxysteroid dehydrogenase involved in regulating plant growth and development. *Plant Physiol.*, **145**, 87–97.

115 Apse, M.P., Aharon, G.S., Snedden, W.A., and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. *Science*, **285**, 1256–1258.

116 Shi, H., Ishitani, M., Kim, C., and Zhu, J.-K. (2000) The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na^+/H^+ antiporter. *Proc. Natl. Acad. Sci. USA*, **97**, 6896–6901.

117 Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L., and Fink, G.R. (1999) The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proc. Natl. Acad. Sci. USA*, **96**, 1480–1485.

118 Zhang, H.-X. and Blumwald, E. (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat. Biotech.*, **19**, 765–768.

119 Zhang, H.-X., Hodson, J.N., Williams, J.P., and Blumwald, E. (2001) Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci. USA*, **98**, 12832–12836.

120 Yang, Q., Chen, Z.-Z., Zhou, X.-F., Yin, H.-B., Li, X., Xin, X.-F., Hong, X.-H., Zhu, J.-K., and Gong, Z. (2009) Overexpression of SOS (Salt Overly Sensitive) genes increases salt tolerance in transgenic *Arabidopsis*. *Mol. Plant.*, **2**, 22–31.

121 Brini, F., Hanin, M., Mezghani, I., Berkowitz, G.A., and Masmoudi, K. (2007) Overexpression of wheat Na^+/H^+ antiporter TNHX1 and H^+-pyrophosphatase TVP1 improve salt- and drought-stress tolerance in *Arabidopsis thaliana* plants. *J. Exp. Bot.*, **58**, 301–308.

122 Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D., and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.*, **110**, 249–257.

123 Rhodes, D. and Hanson, A.D. (2003) Quaternary Ammonium and Tertiary Sulfonium Compounds in Higher Plants. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.*, **44**, 357–384.

124 Makela, P., Peltonen-Sainio, P., Jokinen, K., Pehu, E., Setala, H., Hinkkanen, R., and Somersalo, S. (1996) Uptake and translocation of foliar-applied glycinebetaine in crop plants. *Plant Science*, **121**, 221–230.

125 Krall, J.P., Edwards, G.E., and Andreo, C.S. (1989) Protection of pyruvate, Pi dikinase from maize against cold lability by compatible solutes. *Plant Physiol.*, **89**, 280–285.

126 Zhao, Y., Aspinall, D., and Paleg, L. (1992) Protection of membrane integrity in *Medicago sativa* L. by glycine betaine against the effects of freezing. *J. Plant Physiol.*, **140**, 541–543.

127 Papageorgiou, G. and Murata, N. (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. *Photosynth. Res.*, **44**, 243–252.

128 McCue, K.F. and Hanson, A.D. (1990) Drought and salt tolerance: towards understanding and application. *Trends Biotechnol.*, **8**, 358–362.

129 Landfald, B. and Strom, A.R. (1986) Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. *J. Bacteriol.*, **165**, 849–855.

130 Deshnium, P., Gombos, Z., Nishiyama, Y., and Murata, N. (1997) The action *in vivo* of glycine betaine in enhancement of tolerance of *Synechococcus* sp. strain PCC

7942 to low temperature. *J. Bacteriol.*, **179**, 339–344.

131 Sakamoto, A. and Murata, N. (2000) Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. *J. Exp. Bot.*, **51**, 81–88.

132 Waditee, R., Bhuiyan, M.N.H., Rai, V., Aoki, K., Tanaka, Y., Hibino, T., Suzuki, S., Takano, J., Jagendorf, A., Takabe, T., and Takabe, T. (2005) Genes for direct methylation of glycine provide high levels of glycinebetaine and abiotic-stress tolerance in *Synechococcus* and *Arabidopsis*. *Proc. Nat. Acad. Sci.*, **102**, 1318–1323.

133 Hayashi, H., Alia, Sakamoto, A., Nonaka, H., Chen, T., and Murata, N. (1998) Enhanced germination under high-salt conditions of seeds of transgenic *Arabidopsis* with a bacterial gene (codA) for choline oxidase. *J. Plant Res.*, **111**, 357–362.

134 Prasad, K., Sharmila, P., and Pardha Saradhi, P. (2000) Enhanced tolerance of transgenic *Brassica juncea* to choline confirms successful expression of the bacterial codA gene. *Plant Sci.*, **159**, 233–242.

135 Sakamoto, A. and Murata, N. (2001) The use of bacterial choline oxidase, a glycinebetaine-synthesizing enzyme, to create stress-resistant transgenic plants. *Plant Physiol.*, **125**, 180–188.

136 Huang, J., Hirji, R., Adam, L., Rozwadowski, K.L., Hammerlind, J.K., Keller, W.A., and Selvaraj, G. (2000) Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiol.*, **122**, 747–756.

137 Park, E., Jekni, Z., Pino, M., Murata, N., and Chen, T.H.H. (2007) Glycinebetaine accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic tomato plants against abiotic stress. *Plant Cell Environ.*, **30**, 994–1005.

138 Ahmad, R., Kim, M., Back, K.-H., Kim, H.-S., Lee, H.-S., Kwon, S.-Y., Murata, N., Chung, W.-I., and Kwak, S.-S. (2008) Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses. *Plant Cell Rep.*, **27**, 687–698.

139 Siminovitch, D., Rheaume, B., Pomeroy, M.K., and Lepage, M. (1968) Phospholipid, protein and nucleic acid increases in protoplasm and membrane structures associated with the development of extreme freezing resistance in black locust tree cells. *Cryobiology*, **5**, 202.

140 Withers, L.A. and King, P.J. (1979) Proline: a novel cryoprotectant for the freeze preservation of cultured cells of *Zea mays* L. *Plant Physiol.*, **64**, 675–678.

141 Hare, P.D. and Cress, W.A. (1997) Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul.*, **21**, 79–102.

142 Kavi Kishor, P.B., Hong, Z., Miao, G.-H., Hu, C.A.A., and Verma, D.P.S. (1995) Overexpression of 8′-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.*, **108**, 1387–1394.

143 Liu, J. and Zhu, J.K. (1997) Proline accumulation and salt-stress-induced gene expression in a salt-hypersensitive mutant of *Arabidopsis*. *Plant Physiol.*, **114**, 591–596.

144 Gill, S.S. and Tuteja, N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.*, **48**, 909–930.

145 Neill, S., Desikan, R., and Hancock, J. (2002) Hydrogen peroxide signalling. *Curr. Opin. Plant. Biol.*, **5**, 388–395.

146 Roxas, V.P., Smith, R.K.J., Allen, E.R., and Allen, R.D. (1997) Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nat. Biotech.*, **15**, 988–991.

147 Bowler, C., Slooten, L., Vandenbranden, S., Rycke, R.D., Botterman, J., Sybesma, C., van Montagu, M., and Inze, D. (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J.*, **10**, 1723–1732.

148 Mckersie, B.D., Bowley, S.R., Harjanto, E., and Leprince, O. (1996) Water deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.*, **111**, 1177–1181.

149 Van Camp, W., Capiau, K., Van Montagu, M., Inze, D., and Slooten, L. (1996) Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. *Plant Physiol.*, **112**, 1703–1714.

150 Kasukabe, Y., He, L., Nada, K., Misawa, S., Ihara, I., and Tachibana, S. (2004) Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and upregulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. *Plant Cell Physiol.*, **45**, 712–722.

151 Sen Gupta, A., Heinen, J.L., Holady, A.S., Burke, J.J., and Allen, R.D. (1993) Increased resistance to oxidative stress in transgenic plants that over-express chloroplastic Cu/Zn superoxide dismutase. *Nat. Acad. Sci.*, **90**, 1629–1633.

152 Lee, S.-H., Ahsan, N., Lee, K.-W., Kim, D.-H., Lee, D.-G., Kwak, S.-S., Kwon, S.-Y., Kim, T.-H., and Lee, B.-H. (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. *J. Plant Physiol.*, **164**, 1626–1638.

153 Munnik, T. and Vermeer, J.E.M. (2010) Osmotic stress-induced phosphoinositide and inositol phosphate signalling in plants. *Plant Cell Environ.*, **33**, 655–669.

154 Zhu, J.Q., Zhang, J.T., Tang, R.J., Lv, Q.D., Wang, Q.Q., Yang, L., and Zhang, H.X. (2009) Molecular characterization of ThIPK2, an inositol polyphosphate kinase gene homolog from *Thellungiella halophila*, and its heterologous expression to improve abiotic stress tolerance in *Brassica napus*. *Physiol Plant.*, **136**, 407–425

155 Georges, F., Das, S., Ray, H., Bock, C., Nokhrina, K., Kolla, V.A., and Keller, W. (2009) Over-expression of *Brassica napus* phosphatidylinositol-phospholipase C2 in canola induces significant changes in gene expression and phytohormone distribution patterns, enhances drought tolerance and promotes early flowering and maturation. *Plant Cell Environ.*, **32**, 1664–1681

156 Knight, H. and Knight, M.R. (2001) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *Trends Plant Sci.*, **6**, 262–267.

157 Chinnusamy, V., Zhu, J., and Zhu, J.K. (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci.*, **12**, 444–451.

158 Fowler, S. and Thomashow, M.F. (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell*, **14**, 1675–1690.

159 Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., and Harter, K. (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J.*, **50**, 347–363.

160 Robinson, S. and Parkin, I. (2008) Differential SAGE analysis in *Arabidopsis* uncovers increased transcriptome complexity in response to low temperature. *BMC Genomics*, **9**, 434.

161 Close, T.J., Bhat, P.R., Lonardi, S., Wu, Y., Rostoks, N., Ramsay, L., Druka, A., Stein, N., Svensson, J.T., Wanamaker, S., Bozdag, S., Roose, M.L., Moscou, M.J., Chao, S., Varshney, R.K., Szucs, P., Sato, K., Hayes, P.M., Matthews, D.E., Kleinhofs, A., Muehlbauer, G.J., DeYoung, J., Marshall, D.F., Madishetty, K., Fenton, R.D., Condamine, P., Graner, A., and Waugh, R. (2009) Development and implementation of high-throughput SNP genotyping in barley. *BMC Genomics*, **10**, 582.

162 West, M.A.L., Kim, K., Kliebenstein, D.J., van Leeuwen, H., Michelmore, R.W., Doerge, R.W., and St. Clair, D.A. (2007)

Global eQTL mapping reveals the complex genetic architrave of transcript-level variation in *Arabidopsis*. *Genetics*, **175**, 1441–1450.

163 Hinney, A., Nguyen, T.T., Scherag, A., Friedel, S., Brönner, G. *et al*. (2007) Genome wide association (GWA) study for early onset extreme obesity supports the role of fat mass and obesity associated gene (FTO) variants. *PLoS One*, **2** (12), e1361.

164 Cockram, J., White, J., Zuluaga, D.L., Smith, D., Comadran, J., Macaulay, M., Luo, Z., Kearsey, M.J., Werner, P., Harrap, D., Tapsell, C., Liu, H., Hedley, P.E., Stein, N., Schulte, D., Steuernagel, B., Marshall, D.F., Thomas, W.T., Ramsay, L., Mackay, I., Balding, D.J., Consortium, T.A., Waugh, R., O'Sullivan, D.M., and Members of the AGOUEB Consortium. (2010) Genome-wide association mapping to candidate polymorphism resolution in the unsequenced barley genome. *Proc. Natl. Acad. Sci. USA*, **107** (50), 21611–21616.

165 Welch, R.M. and Graham, R.D. (1999) A new paradigm for world agriculture: meeting human needs: productive, sustainable, nutritious. *Field Crops. Res.*, **60**, 1–10.

166 Newton, A.C., Akar, T., Baresel, J.P., Bebeli, P.J., Bettencourt, E., Bladenopoulos, K.V., Czembor, J.H., Fasoula, D.A., Katsiotis, A., Koutis, K., Koutsika-Sotiriou, M., Kovacs, G., Larsson, H., de Carvalho, M.A.A.P., Rubiales, D., Russell, J., Dos Santos, T.M.M., and Vaz Patto, M.C. (2010) Cereal landraces for sustainable agriculture. A review. *Agron. Sustain. Dev.*, **30**, 237–269.

167 McVetty, P.B.E. and Scarth, R. (2002) Breeding for imporved oil quality in *Brassica* oilseed species. *J. Crop Production*, **5**, 345–369.

168 Zhao, J., Becker, H.C., Zhang, D., Zhang, Y., and Ecke, W. (2005) Oil content in a European×Chinese rapeseed population: QTL with additive and epistatic effects and their genotype–environment interactions. *Crop Sci.*, **45**, 51–59.

169 Foisset, N., Delourme, R., Barret, P., and Renard., M. (1995) Molecular tagging of the dwarf BREIZH (Bzh) gene in *Brassica napus*. *Theor. Appl. Genet.*, **91**, 756–761.

170 Child, R.D., Summers, J.E., Babij, J., Farrent, J.W., and Bruce, D.M. (2003) Increased resistance to pod shatter is associated with changes in the vascular structure in pods of a resynthesized *Brassica napus* line. *J. Exp. Bot.*, **54**, 1919–1930.

171 Østergaard, L., Kempin, S.A., Bies, D., Klee, H.J., and Yanofsky, M.F. (2006) Pod shatter-resistant *Brassica* fruit produced by ectopic expression of the FRUITFULL gene. *Plant Biotechnol. J.*, **4**, 45–51.

172 Wang, R., Ripley, V.L., and Rakow, G. (2007) Pod shatter resistance evaluation in cultivars and breeding lines of *Brassica napus*, *B. juncea* and *Sinapis alba*. *Plant Breed*, **126**, 588–595.

173 Rimmer, S.R. and van den Berg, C.G.J. (1992) Resistance of oilseed *Brassica* spp. to blackleg caused by *Leptosphaeria maculans*. *Can. J. Plant Pathol.*, **14**, 56–66.

174 Thurling, N. and Depittayanan, V. (1992) EMS induction of early flowering mutants in spring rape (*Brassica napus*). *Plant Breed*, **108**, 177–184.

175 Li, C.X. and Cowling, W.A. (2003) Identification of a single dominant allele for resistance to blackleg in *Brassica napus* 'Surpass 400'. *Plant Breed.*, **122**, 485–488.

176 Blum, A. (1988) *Plant Breeding for Stress Environments*, CRC Press, Boca Raton, FL (USA).

177 Mittler, R. (2006) Abiotic stress, the field environment and stress combination. *Trends Plant Sci.*, **11**, 15–19.

178 Passioura, J. (2007) The drought environment: physical, biological and agricultural perspectives. *J. Exp. Bot.*, **58**, 113–117.

179 Murray, M.B., Cape, J.N., and Fowler, D. (1989) Quantification of frost damage in plant tissues by rates of electrolyte leakage. *New Phytol.*, **113**, 307–311.

180 Griffith, M. and Mclntyre, H.C.H. (1993) The interrelationship of growth and frost tolerance in winter rye. *Physiol. Plant.*, **87**, 335–344.

181 Teulat, B., Zoumarou-Wallis, N., Rotter, B., Ben Salem, M., Bahri, H., and This, D. (2003) QTL for relative water content in field-grown barley and their

stability across Mediterranean environments. *Theor. Appl. Genet.*, **108**, 181–188.

182 Wooten, D.R., Livingston, D.P., III, Lyerly, H.J., Holland, J.B., Jellen, E.N., Marshall, D.S., and Murphy, J.P. (2009) Quantitative trait loci and epistasis for oat winter-hardiness component traits. *Crop. Sci.*, **49**, 1989–1998.

183 Sanguineti, M., Tuberosa, R., Landi, P., Salvi, S., Maccaferri, M., Casarini, E., and Conti, S. (1999) QTL analysis of drought-related traits and grain yield in relation to genetic variation for leaf abscisic acid concentration in field-grown maize. *J. Exp. Bot.*, **50**, 1289–1297.

184 Havaux, M. and Lannoye, R. (1983) Chlorophyll fluorescence induction: a sensitive indicator of water stress in maize plants. *Irrigation Sci.*, **4**, 147–151.

185 Havaux, M. and Lannoye, R. (1985) Drought resistance of hard wheat cultivars measured by a rapid chlorophyll fluorescence test. *J. Agr. Sci.*, **104**, 501–504.

186 Ribaut, J.-M. and Ragot, M. (2007) Marker-assisted selection to improve drought adaptation in maize: the backcross approach, perspectives, limitations, and alternatives. *J. Exp. Bot.*, **58**, 351–360.

187 Kianian, S.F. and Quiros, C.F. (1992) Generation of a *Brassica oleracea* composite RFLP map: linkage arrangements among various populations and evolutionary implications. *Theor. Appl. Genet.*, **84**, 544–554.

188 Teutonico, R.A. and Osborn, T.C. (1994) Mapping of RFLP and qualitative trait loci in *Brassica rapa* and comparison to the linkage maps of *B. napus*, *B. oleracea*, and *Arabidopsis thaliana*. *Theor. Appl. Genet.*, **89**, 885–894.

189 Parkin, I.A.S., Keith, A.G., and Lydiate, D.J. (1995) Identification of the A and C genomes of amphidiploid *Brassica napus* (oilseed rape). *Genome*, **38**, 1122–1131.

190 Uzunova, M., Ecke, W., Weissleder, K., and Röbbelen, G. (1995) Mapping the genome of rapeseed (*Brassica napus* L.). I. Construction of an RFLP linkage map and localization of QTLs for seed glucosinolate content. *Theor. Appl. Genet.*, **90**, 194–204.

191 Foisset, N., Delourme, R., Barret, P., Hubert, N., Landry, B.S., and Renard, M. (1996) Molecular-mapping analysis in *Brassica napus* using isozyme, RAPD and RFLP markers on a doubled-haploid progeny. *Theor. Appl. Genet.*, **93**, 1017–1025.

192 Lagercrantz, U. and Lydiate, D.J. (1996) Comparative genome mapping in *Brassica*. *Genetics*, **144**, 1903–1910.

193 Axelsson, T., Bowman, C.M., Sharpe, A.G., Lydiate, D.J., and Lagercrantz, U. (2000) Amphidiploid *Brassica juncea* contains conserved progenitor genomes. *Genome*, **43**, 679–688.

194 Pradhan, A., Gupta, V., Mukhopadhyay, A., Arumugam, N., Sodhi, Y., and Pental, D. (2003) A high-density linkage map in *Brassica juncea* (Indian mustard) using AFLP and RFLP markers. *Theor. Appl. Genet.*, **106**, 607–614.

195 Nelson, M.N. and Lydiate, D.J. (2006) New evidence from *Sinapis alba* L. for ancestral triplication in a crucifer genome. *Genome*, **49**, 230–238.

196 Kole, C., Thormann, C.E., Karlsson, B.H., Palta, J.P., Gaffney, P., Yandell, B., and Osborn, T.C. (2002) Comparative mapping of loci controlling winter survival and related traits in oilseed *Brassica rapa* and *B. napus*. *Mol. Breed.*, **9**, 201–210.

197 Asghari, A., Mohammadi, S.A., Moghaddam, M., and Mohammaddoost, H. (2007) Identification of QTLs controlling winter survival in *Brassica napus* using rapid markers. *Biotechnol. Biotechnol.*, **21**, 4.

198 Xin, Z. and Browse, J. (1998) Eskimo1 mutants of *Arabidopsis* are constitutively freezing-tolerant. *Proc. Natl Acad. Sci. USA*, **95**, 7799–7804.

199 Xin, Z., Mandaokar, A., Chen, J., Last, R.L., and Browse, J. (2007) *Arabidopsis* ESK1 encodes a novel regulator of freezing tolerance. *Plant J.*, **49**, 786–799.

200 McKown, R., Kuroki, G., and Warren, G. (1996) Cold responses of *Arabidopsis* mutants impaired in freezing tolerance. *J. Exp. Bot.*, **47**, 1919–1925.

201 Thorlby, G., Fourrier, N., and Warren, G. (2004) The SENSITIVE TO FREEZING2 GENE, required for freezing tolerance in *Arabidopsis thaliana*, encodes a {beta}-Glucosidase. *Plant Cell*, **16**, 2192–2203.

202 Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J.K. (1997) Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell*, **9**, 1935–1949.

203 Guo, Y., Xiong, L., Ishitani, M., and Zhu, J.K. (2002) An *Arabidopsis* mutation in translation elongation factor 2 causes superinduction of CBF/DREB1 transcription factor genes but blocks the induction of their downstream targets under low temperatures. *Proc. Natl. Acad. Sci. USA*, **99**, 7786–7791.

204 Zhu, J., Verslues, P.E., Zheng, X., Lee, B.H., Zhan, X., Manabe, Y., Sokolchik, I., Zhu, Y., Dong, C.H., Zhu, J.K., Hasegawa, P.M., and Bressan, R.A. (2005) HOS10 encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants. *Proc. Natl. Acad. Sci.*, **102**, 9966–9971.

205 Zhuang, J., Xiong, A.-S., Peng, R.-H., Gao, F., Zhu, B., Zhang, J., Fu, X.-Y., Jin, X.-F., Chen, J.-M., Zhang, Z., Qiao, Y.-S., and Yao, Q.-H. (2009) Analysis of *Brassica rapa* ESTs: gene discovery and expression patterns of AP2/ERF family genes. *Mol. Biol. Rep.*, **37** (5), 2485–2492.

206 Qiu, Q.-S., Guo, Y., Dietrich, M.A., Schumaker, K.S., and Zhu, J.-K. (2002) Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proc. Natl. Acad. Sci. USA*, **99**, 8436–8441.

207 Chinnusamy, V., Schumaker, K., and Zhu, J.-K. (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J. Exp. Bot.*, **55**, 225–236.

208 Shinozaki, K. and Yamaguchi-Shinozaki, K. (2007) Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.*, **58**, 221–227.

209 Kumar, S.V. and Wigge, P.A. (2010) H2A.Z-containing nucleosomes mediate the thermosensory response in *Arabidopsis*. *Cell*, **140**, 136–147.

210 Sunkar, R., Chinnusamy, V., Zhu, J., and Zhu, J.-K. (2007) Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends Plant. Sci.*, **12**, 301–309.

211 Mohapatra, S.S., Wolfraim, L., Poole, R.J., and Dhindsa, R.S. (1989) Molecular cloning and relationship to freezing tolerance of cold-acclimation-specific genes of Alfalfa. *Plant Physiol.*, **89**, 375–380.

212 Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. (2001) Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell*, **13**, 61–72.

213 Chen, T.H.H. and Murata, N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.*, **5**, 250–257.

214 Hudson, M.E., Bruggink, T., Chang, S.H., Yu, W., Han, B., Wang, X., van der Toorn, P., and Zhu, T. (2007) Analysis of gene expression during *Brassica* seed germination using a cross-species microarray platform. *Crop Sci.*, **47**, S– 96-S-112.

215 Lee, S., Lim, M., Kim, J., Lee, S., Kim, J., Jin, M., Kwon, S., Mun, J., Kim, Y., Kim, H., Hur, Y., and Park, B. (2008) Transcriptome analysis in *Brassica rapa* under the abiotic stresses using *Brassica* 24K oligo microarray. *Mol. Cells*, **26**, 595–605.

216 Xiang, D., Datla, R., Li, F., Cutler, A., Malik, M.R., Krochko, J.E., Sharma, N., Fobert, P., Georges, F., Selvaraj, G., Tsang, E., Klassen, D., Koh, C., Deneault, J.S., Nantel, A., Nowak, J., Keller, W., and Bekkaoui, F. (2008) Development of a *Brassica* seed cDNA microarray. *Genome*, **51** 236–242.

217 Robinson, S.J., Cram, D.J., Lewis, C.T., and Parkin, I.A.P. (2004) Maximizing the efficacy of SAGE analysis identifies novel transcripts in *Arabidopsis*. *Plant Physiol.*, **136**, 3223–3233.

218 Trick, M., Cheung, F., Drou, N., Fraser, F., Lobenhofer, E.K., Hurban, P., Magusin,

A., Town, C.D., and Ian, B. (2009) A newly-developed community microarray resource for transcriptome profiling in *Brassica* species enables the confirmation of *Brassica*-specific expressed sequences. *BMC Plant Biol.*, **9**, 50.

219 Parkin, I.A.P., Gulden, S.M., Sharpe, A.G., Lukens, L., Trick, M., Osborn, T.C., and Lydiate, D.J. (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. *Genetics*, **171**, 765–81.

220 Town, C.D., Cheung, F., Maiti, R., Crabtree, J., Haas, B.J., Wortman, J.R., Hine, E.E., Althoff, R., Arbogast, T.S., Tallon, L.J., Vigouroux, M., Trick, M., and Bancroft, I. (2006) Comparative genomics of *Brassica oleracea* and *Arabidopsis thaliana* reveal gene loss, fragmentation, and dispersal after polyploidy. *Plant Cell*, **18**, 1348–1359.

221 Mun, J.H., Kwon, S.J., Seol, Y.J., Kim, J.A., Jin, M., Kim, J.S., Lim, M.H., Lee, S.I., Hong, J.K., Park, T.H., Lee, S.C., Kim, B.J., Seo, M.S., Baek, S., Lee, M.J., Shin, J.Y., Hahn, J.H., Hwang, Y.J., Lim, K.B., Park, J.Y., Lee, J., Yang, T.J., Yu, H.J., Choi, I.Y., Choi, B.S., Choi, S.R., Ramchiary, N., Lim, Y.P., Fraser, F., Drou, N., Soumpourou, E., Trick, M., Bancroft, I., Sharpe, A.G., Parkin, I.A., Batley, J., Edwards, D., and Park, B.S. (2010) Sequence and structure of *Brassica rapa* chromosome A3. *Genome Biol.*, **11** (9) R94.

222 Brassica rapa Genome Sequencing Project Consortium. (2011). The genome of the mesopolyploid crop species *Brassica rapa*. *Nat Genet.* **43**: 1035–1039.

223 Brachi, B., Faure, N., Horton, M., Flahauw, E., Vazquez, A., Nordborg, M., Bergelson, J., Cuguen, J., and Roux, F. (2010) Linkage and association mapping of *Arabidopsis thaliana* flowering time in nature. *PLoS Genet.*, **6**, e1000940.

224 Nemri, A., Atwell, S., Tarone, A.M., Huang, Y.S., Zhao, K., Studholme, D.J., Nordborg, M., and Jones, J.D. (2010) Genome-wide survey of *Arabidopsis* natural variation in downy mildew resistance using combined association and linkage mapping. *Proc. Natl. Acad. Sci. USA*, **22**, 10302–10307.

225 Myles, S., Peiffer, J., Brown, P.J., Ersoz, E.S., Zhang, Z., Costich, D.E., and Buckler, E.S. (2009) Association mapping: critical considerations shift from genotyping to experimental design. *Plant Cell*, **21**, 2194–2202.

226 Trick, M., Long, Y., Meng, J., and Bancroft, I. (2009) Single nucleotide polymorphism (SNP) discovery in the polyploid *Brassica napus* using Solexa transcriptome sequencing. *Plant Biotechnol. J.*, **7**, 334–346.

227 Druka, A., Potokina, E., Luo, Z., Jiang, N., Chen, X., Kearsey, M., and Waugh, R. (2010) Expression quantitative trait loci analysis in plants. *Plant Biotechnol. J.*, **8** (1), 10–27.

228 Marilyn, A.L., West, M.A.L., Kim, K., Kliebenstein, D.J., van Leeuwen, H., Michelmore, R.W., Doerge, R.W., Dina, A., and St. Clair, D.A. (2007) Global eQTL mapping reveals the complex genetic architecture of transcript-level variation in *Arabidopsis*. *Genetics*, **175**, 1441–1450.

229 Chen, X., Hackett, C.A., Niks, R.E., Hedley, P.E., Booth, C., Druka, A., Marcel, T.C., Vels, A., Bayer, M., Milne, I., Morris, J., Ramsay, L., Marshall, D., Cardle, L., and Waugh, R. (2010) An eQTL analysis of partial resistance to *Puccinia hordei* in barley. *PLoS One*, **5**, e8598.

230 Jansen, R.C., Tesson, B.M., Fu, J., Yang, Y., and McIntyre, L.M. (2009) Defining gene and QTL networks. *Curr. Opin. Plant Biol.*, **12**, 241–246.

231 Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNA from *Arabidopsis*. *Plant Cell*, **16**, 2001–2019.

232 Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R., and Zhu, J.K. (2005) Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell*, **123**, 1279–1291.

233 Zhang, X., Yazaki, J., Sundaresan, A., Cokus, S., Chan, S.W., Chen, H., Henderson, I.R., Shinn, P., Pellegrini, M., Jacobsen, S.E., and Ecker, J.R. (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in *Arabidopsis*. *Cell*, **126**, 1189–1201.

234 Suzuki, M.M. and Bird, A. (2008) DNA methylation landscapes: provocative insights from epigenomics. *Nature Reviews Genetics*, **9**, 465–476.

235 King, G.J., Amoah, S., and Kurup, S. (2010) Exploring and exploiting epigenetic variation in crops. *Genome*, **53**, 856–868.

236 Friedt, W., Snowdon, R., Ordon, F., and Ahlemeyer, J. (2007) *Plant Breeding: Assessment of Genetic Diversity in Crop Plants and its Exploitation in Breeding*, Springer, Berlin.

237 Becker, H.C., Engqvist, G.M., and Karlsson, B. (1995) Comparison of rapeseed cultivars and resynthesised lines based on allozyme and RFLP markers. *Theor. Appl. Genet.*, **91**, 62–67.

238 Gehringer, A., Snowdon, R., Spiller, T., Basunanda, P., and Friedt, W. (2007) New oilseed rape (*Brassica napus*) hybrids with high levels of heterosis for seed yield under nutrient-poor conditions. *Breed. Sci.*, **57**, 315–320.

239 Sayis, F., Snowdon, R.J., Luhs, W., and Friedt, W. (2003) Molecular characterisation of novel resynthesized rapeseed (*Brassica napus* L.) lines and analysis of their genetic diversity in comparison to spring rapeseed cultivars. *Plant Breed.*, **122**, 473–478.

240 Stephenson, P., Baker, D., Girin, T., Perez, A., Amoah, S., King, G.J., and Østergaard, L. (2010) A rich TILLING resource for studying gene function in *Brassica rapa*. *BMC Plant Biol.*, **10**, 62.

241 Mohan, J.S. (2001) Tissue culture-derived variation in crop improvement. *Euphytica*, **118**, 153–166.

242 Snowdon, R.J., Winter, H., Diestel, A., and Sacristán, M.D. (2000) Development and characterisation of *Brassica napus–Sinapis arvensis* addition lines exhibiting resistance to *Leptosphaeria maculans*. *Theor. Appl. Genet.*, **101**, 1008–1014.

243 Srivastava, A., Mukhopadhyay, A., Arumugam, N., Gupta, V., Verma, J.K., Pental, D., and Pradhan, A.K. (2004) Resynthesis of *Brassica juncea* through interspecific crosses between *B. rapa* and *B. nigra*. *Plant Breed.*, **123**, 204–206.

244 Wong, C., Li, Y., Whitty, B., Díaz-Camino, C., Akhter, S., Brandle, J., Golding, G., Weretilnyk, E., Moffatt, B., and Griffith, M. (2005) Expressed sequence tags from the Yukon ecotype of *Thellungiella* reveal that gene expression in response to cold, drought and salinity shows little overlap. *Plant Mol. Biol.*, **58**, 561–574.

245 Jin, D., Yue-Ping, H., Jing, X., Min-Jie, C., Wan-Song, N., Xi, C., Jian-Kang, Z., David, J.O., and Cheng-Bin, X. (2008) Functional gene-mining for salt-tolerance genes with the power of *Arabidopsis*. *Plant J.*, **56**, 653–664.

246 Kant, S., Bi, Y.-M., Weretilnyk, E., Barak, S., and Rothstein, S.J. (2008) The *Arabidopsis* halophytic relative *Thellungiella halophila* tolerates nitrogen-limiting conditions by maintaining growth, nitrogen uptake, and assimilation. *Plant Physiol.*, **147**, 1168–1180.

247 Wu, C., Gao, X., Kong, X., Zhao, Y., and Zhang, H. (2009) Molecular cloning and functional analysis of a Na^+/H^+ antiporter gene ThNHX1 from a halophytic plant *Thellungiella halophila*. *Plant Mol. Biol. Rep.*, **27**, 1–12.

248 Pedras, M.S.C., Chumala, P.B., and Suchy, M. (2003) Phytoalexins from *Thlaspi arvense*, a wild crucifer resistant to virulent *Leptosphaeria maculans*: structures, syntheses and antifungal activity. *Phytochemistry*, **64**, 949–956.

249 Sharma, N., Cram, D., Huebert, T., Zhou, N., and Parkin, I. (2007) Exploiting the wild crucifer *Thlaspi arvense* to identify conserved and novel genes expressed during a plant's response to cold stress. *Plant Mol. Biol.*, **63**, 171–184.

250 Zhou, N., Robinson, S., Huebert, T., Bate, N., and Parkin, I. (2007) Comparative genome organization reveals a single copy of CBF in the freezing tolerant crucifer *Thlaspi arvense*. *Plant Mol. Biol.*, **65**, 693–705.

52
Mustard: Approaches for Crop Improvement and Abiotic Stress Tolerance

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Adverse environmental conditions seriously affect crop growth, productivity, and genome stability. These adverse environmental factors are a menace for plants that prevent them from reaching their full genetic potential and therefore limit the crop productivity worldwide. Stress conditions, such as extreme temperatures, water availability, and ion toxicity, represent abiotic stresses, which cause massive loss of crop yield. Global climatic pattern is also becoming more unpredictable with increased occurrence of drought, flood, storms, heat waves, and seawater intrusion. The oleiferous *Brassica* is the third most important source of vegetable oil in the world after palm and soybean oil and grown as an edible or an industrial oil crop that is used as a source of edible protein, in much the same way as soybean protein. According to a report of the United States Department of Agriculture (USDA), the world oilseed production was 397 metric ton in 2006–2007 and Indian agriculture contributed about 15 and 8% to the world total acreage under oilseed cultivation and production, respectively. However, the average productivity in India is only 791 kg ha^{-1} compared to the world average of 1718 kg ha^{-1}. Despite a large area under cultivation of mustard, the productivity of the crop has dropped in recent years because plant growth and development are affected by various abiotic stress factors. Protecting crop productivity under unfavorable environmental conditions is a major challenge for modern agriculture. In this review, we have attempted to provide an overview of success obtained in raising germplasm with improved salinity tolerance through breeding methods. The recent "Omics" approaches and their applications in abiotic stress research on mustard crop are also presented.

52.1
Introduction

Environmental stress is referred to the extreme environmental conditions that lead to alterations in plant metabolism resulting in decreased rate of plant's growth

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processes, loss in productivity, or inducing damaging effect on any of plant organs/parts besides alteration in anatomical, biochemical, or molecular regulation. Abiotic stresses such as extreme temperatures (freezing, cold, and heat), water availability (drought and flooding), and ion toxicity (salinity, alkalinity, and heavy metals) are the major causes that adversely affect the plant growth and productivity worldwide [1–5]. It has been found that the relative decreases in potential yields associated with abiotic stress factors vary between 54 and 82% [2, 6]. It is estimated from the comparison of record yields and average yields for various crop plants that crops mainly attain only 20% of their genetic potential for yield due to various biotic and abiotic stress factors. Therefore, it is clear that there is an urgent need to increase abiotic stress tolerance in plants. Increasing crop production is now the highest agricultural priority worldwide because of increasing world population. According to the United Nation's World Population Prospects Report, the world population is increasing at an alarming rate of approximately 74 million people per year and is expected to reach more than 9 billion by 2050 (http://www.un.org/esa/population/unpop.htm), whereas global food productivity is declining due to the negative effects of various environmental stress factors.

Plants respond to various stress factors such as salinity, heat, cold, drought, excess water, heavy metal toxicity, wounding, excess light, nutrient loss, anaerobic conditions, and radiations through multifaceted molecular signaling pathways by perceiving and transducing the stress signal(s) through a cascade of molecular networks eventually leading to the expression of stress-related genes. These responses at the molecular, cellular, physiological and biochemical levels enable the plants to survive [7, 8]. These responses include effects of abiotic stresses on overall growth and development, electrolyte leakage, osmolyte accumulation, and the ion homeostasis [8]. The new molecular "omics" tools have opened up new perspectives in stress biology. The "omics" approaches such as genomics, proteomics, metabolomics, and transcriptomics have direct potential for improving abiotic stress tolerance in crop plants that require knowledge of stress response at molecular level, which includes gene expression to protein or metabolite and its phenotypic effects. Integration of phenotypic, genomic, transcriptomic, proteomic, and metabolomic data will enable accurate and detailed gene network reconstruction.

The oleiferous *Brassica* play an important role in global agriculture and horticulture. These crops contribute both to the economy and to the health of populations around the world. *Brassica* is the third most important source of vegetable oil in the world after palm and soybean oil and is grown as an edible or an industrial oil crop that is used as a source of edible protein, in much the same way as soybean protein [9]. *Brassica* is the Latin name of a genus that is taxonomically placed within the Brassicaceae (Cruciferae), which is one of the 10th most economically important plant families in the world. The major mustard oil-producing countries include Canada, China, France, Germany, India, and the United Kingdom. According to a report of the United States Department of Agriculture (USDA), the world oilseed production was 397 metric ton in 2006–2007. Indian agriculture

contributes about 15 and 8% to the world's total acreage under oilseed cultivation and production, respectively. However, the productivity in India is only 791 kg ha^{-1} compared to the world average of 1718 kg ha^{-1} [10]. About 90% of the total land under oilseed cultivation in India is occupied by *Brassica juncea* [11]. Indian mustard (*B. juncea* L. Czern & Coss.), belonging to the same genus *Brassica* in the Cruciferae family, is an agriculturally and economically important crop widely cultivated in Asia and Europe, and abiotic stress is a major constraint to its productivity. *B. juncea* is a dominant crop grown for human consumption as condiments, fodder, and seed. The *Brassica* plant is characterized by a large number of broad, oblong-shaped leaves present in the lower layers of the plant axis [12]. *B. juncea* is a more productive oilseed than *B. napus* in hot regions of Russia, India, China, and Canada with somewhat unreliable rainfalls, whereas *B. napus* is the higher yielding species in more temperate, wetter regions. Oil of *B. juncea* is consumed in large quantity and the production ranks second among all oilseeds in India [13]. In India, *B. juncea* is used as oil-bearing crop and has been studied extensively [14, 15]. However, due to the presence of undesirable long-chain fatty acids such as ecosenoic acid (10%) and erucic acid (50%) in the seed oil, it becomes detrimental to human health. Erucic acid increases blood cholesterol, interferes with myocardial conductance, and shortens coagulation time [16]. European Economic Commission has restricted cultivation of *Brassica* crop that contains more than 10% erucic acid content in their oil [17]. The discovery of the genes for low erucic acid oil production in the seeds of Indian mustard began the conversion of this ancient crop to a canola-type oilseed for dry areas.

52.1.1
Description of Indian Mustard

B. juncea (Indian mustard) belongs to the family Cruciferae. There are nearly 40 different varieties of this yellow flowering plant that are classified by the botanists into the genus *Brassica* of which *B. juncea* is one of the major oilseed crops of India. The oil content varies between 35 and 45% and the protein content is between 20 and 24%. The seed residue is used as cattle feed and in fertilizers. It is a high biomass crop characterized by oblong-shaped leaves.

52.1.2
Origin of Indian Mustard

Studies suggest that *Brassica* evolved from the genus *Sinapidendron* of the Miocene age through *Diplotaxis–Erucastrum* complex [18]. *B. juncea* is generally thought to have originated in the Middle East, whereas *B. rapa* and *B. nigra* species overlapped in the wild, but Central Asia and China are suggested as the sites of primary origin [19]. Hemingway [20], however, considers that *B. juncea* (L.) may also have arisen by independent hybridization at secondary centers in India, China, and Caucasus, as *B. nigra* was widely used as the commercial spice from early times.

52.1.3 Nomenclature

The oleiferous *Brassica* grown in India are divided into the following four groups:

1) Brown mustard: Commonly known as rai, raya, or laha (*B. juncea* L. Czern & Coss.)
2) Sarson
   a) Yellow sarson: *B. campestris* L. var. Sarson Prain
   b) Brown sarson: *B. campestris* L. var. Dichotoma Watt
3) Toria: lahi or maghi lahi (*B. campestris* L. var. Toria Duth)
4) Taramira or Tara (*Eruca sativa* Mill.)

In addition, there are two other species, namely, *B. nigra* Koch. (Banarasi rai) and *B. juncea* var. Rugosa (Pahadi rai). These two species do not fall under any of the four groups. These are, moreover, grown to a limited extent. Mustard (*B. juncea* L. Czern & Coss.) is the dominant species grown in India [19].

52.1.4 Botanical Description

Rape and mustard include annual herbs. Roots, in general, are long and tapering. Toria is more or less a surface feeder but Brown sarson bears long roots with limited lateral spread enabling its successful cultivation under drier conditions. The height of the stem varies from 45 cm (in some varieties of Toria) to 190 cm (in Yellow sarson). In Toria and Brown sarson, the branches arise at an angle of 30–40°. In Yellow sarson, the branches arise laterally at an angle of about 10–20° giving the plant a narrow and pyramidal shape. The inflorescence is a corymbose raceme. In the case of yellow sarson, the four petals are spread apart, whereas, in brown sarson and Toria, the petals overlap or may be placed apart, depending upon the cultivar. The flowers bear a hypogynous ovary. In Brown sarson and Toria, the ovary is bicarpellary, whereas in Yellow sarson, it may also be tri- or tetracarpellary. The fruit is sliliqua. The sliliqua are two-valved, three-valved, or four-valved, depending upon the number of carpels in the ovary. The flowers begin to open from 8:00 h and continue up to 12:00 h noon.

52.1.5 Genomic Relationships of *B. juncea*

The relationships among the cultivated *Brassica* species were largely clarified by cytological work. There are six species of *Brassica* that merit attention for their economic importance. Among the six species, three are diploid, *B. campestris* ($2n = 20$, AA), *B. nigra* ($2n = 16$, BB), and *B. oleraceae* ($2n = 18$, CC), and the other three are amphidiploids, *B. juncea* ($2n = 36$, AABB), *B. napus* ($2n = 38$, AACC), and *B. carinata* ($2n = 34$, BBCC). The botanical and genomic relationships between these six species may be represented in the form of a triangle usually known as triangle of U [21] (Figure 52.1).

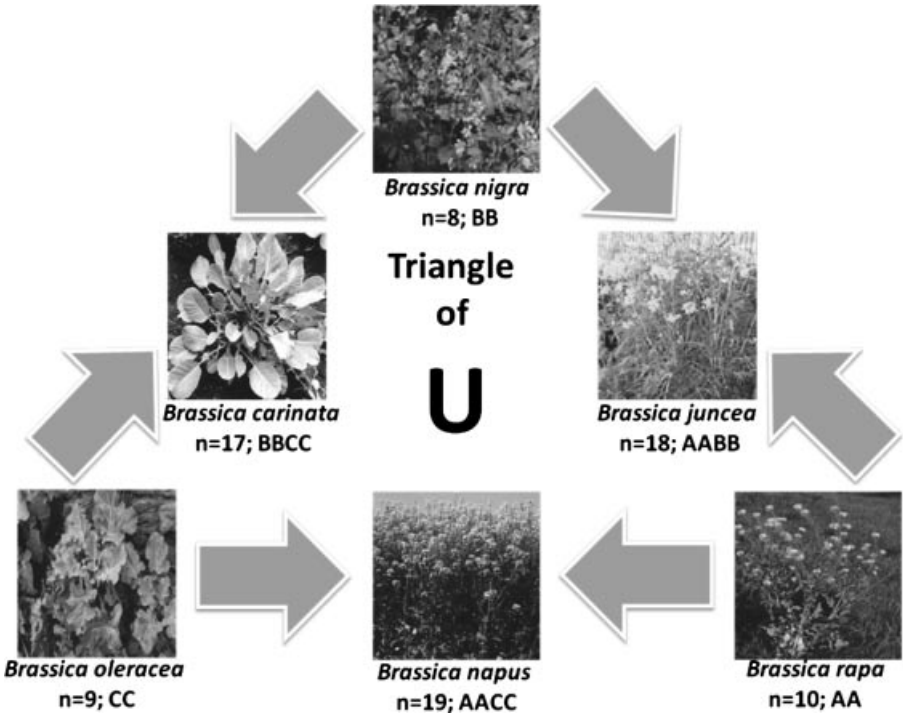

Figure 52.1 "Triangle of U" representing the genomic relationships among six crop species of *Brassica*. Three diploid species are shown (*B. rapa*, *B. nigra*, and *B. oleracea*), which represent the AA, BB, and CC genomes, respectively. Also shown are three tetraploid species (*B. carinata*, *B. juncea*, and *B. napus*), which are hybrid combinations of the basic genomes.

In this chapter, we have tried to explore the available literature on "omics" approaches such as genomics, transcriptomics, proteomics, and metabolomics in *Brassica*, the most important oilseed crop, for possible crop improvement and abiotic stress tolerance. We also present the success obtained so far in improving the salinity tolerance in brassicas employing the traditional breeding approaches.

52.2
Indian Mustard: Breeding Aspects under Salinity Stress

Limited efforts have been made for improving crop varieties for various abiotic stresses following conventional selection and breeding programs. The complex mechanisms of abiotic stress tolerance, characterized by the expression of several minor genes limit the rate and extent of progress in improving stress tolerance in crops through conventional breeding program. Furthermore, techniques employed for selecting tolerant plants are time consumable and consequently expensive. Drought and salinity are the serious impediments in decreasing the growth and yield performance of different crop varieties. The availability of genetic resources

besides the creation and exploitation of novel variability forms the basis of conventional breeding approaches. Cultivars with improved performance under different abiotic stresses have been developed through selection and evaluation in the targeted drought-and salinity-affected areas. Concerted breeding efforts at Central Soil Salinity Research Institute have resulted in the development and release of salinity tolerant cultivars such as CS52, CS54, and CS56. These varieties have consistently performed better in the salt-affected soils than the national checks, Varuna and Kranti. Further studies have shown that amphipolyploid species, namely, *B. napus*, *B. carinata*, and *B. juncea* are more tolerant toward salinity than the diploid brassicas. *B. napus* followed by *B. carinata* have been characterized as more salt tolerant, particularly in climatic zones with long cool periods. *B. juncea*, probably due to its shorter growth period compared to *B. napus* and *B. carinata*, has proved more promising in semiarid and arid climates with short winters. Brassicas are by and large more vulnerable to alkalinity than to salinity. Mustard genotypes have shown tremendous variability in plant types. Vigorous growth during vegetative stage, medium plant height, early maturity, and better response to added inputs are some of the desired characteristics, which are associated with consistent yield performance over an array of environments. Genotypes with medium plant height appear to be more stable in varied environments. The exploitation of hybrid vigor, through the use of CMS and appropriate restorer, will go a long way in raising the production potential of rapeseed and mustard in India.

Besides the yield potential and stability, germplasm is improved for traits contributing to increased productivity. Physiological characters such as nutrient use efficiency and harvest index need attention. The rapeseed–mustard crops produce large biomass but fail to translocate it to the sink, resulting in low harvest index. Attempts should also be made to breed varieties for better response to low nitrogen application, which needs tailoring of the new plant type having less secondary and tertiary branching, reduced height, longer main shoot, more siliqua on main shoot, more number of seeds/siliqua, and higher 1000-seed weight with better nutrient use efficiency and capacity to transport assimilate to seeds.

Salinity stress causes yield decline in all the species and the genotypes. The challenge is to improve the genotypes with salt tolerance characteristics retaining the economic potential of the particular genotype. Stressful environments are often said to increase the expansion of inbreeding depression. Plant's behavior also varies with intensity of stress. Inbreeding depression occurs for survival under high stress conditions and for growth under lower stress levels. The workable strategy lies in selection for tolerant genotypes paying lesser penalty in terms of yield decline. A large number of germplasm needs to be evaluated under target environments to identify the breeding material.

52.2.1 Screening Methodology for Seedling Emergence

Under field salinity conditions, it has been observed that once a seedling emerges, it continues to grow with relatively lesser reductions in growth and yield. Accordingly, it

is imperative to know the salt tolerance potential of different Indian mustard genotypes for seedling emergence by some rapid screening methodology under laboratory conditions. For this, different genotypes have been evaluated under solution culture in laboratory, sand culture, and soil culture in pots to know their interrelationships. Variability in different genotypes with respect to their behavior under salinity stress may be attributed to the differences in uptake of toxic ions along with the absorption of water by the seeds. Increase in Na^+ uptake along with the decline in K^+ uptake under salinity stress during germination has been reported by Sharma [22]. Furthermore, seedling emergence under solution culture and sand culture declined only due to the ionic stress caused by salinity of the soil solution, whereas in soil culture, osmotic effects play an associative role along with ionic effects. Accordingly, the higher decline in seedling emergence under soil culture may be associated with aggravation in ionic accumulation caused by the increasing osmotic stress along with matric stress. Seedling emergence under soil culture was also observed to decline through post-germination salt injuries to hypocotyls at the time of their protruding through soil.

Application of piece-wise linear response model to the pooled data of seven genotypes in different types of growing medium showed that threshold salinity level was highest in solution culture experiments ($C_t = 14.15$). It declined to 7.84 in sand culture and further to 5.96 in soil culture experiments [23]. Similarly, the salinity level at which 50% reduction in seedling emergence occurred was also highest in solution culture ($C_{50} = 21.06$), which declined to 15.96 in sand culture and to 12.31 in soil culture experiments. Along with the decline in C_t and C_{50} upon shifting from solution to sand and soil culture, the slope values increased from 7.25 in solution culture to 9.30 in soil culture experiments. The regression equation for the pooled data in three types of growing medium was computed to be $Y = -3.8789\ X + 111.417$ ($r^2 = 0.69$). Furthermore, the regression equation for seedling emergence under different salinity levels in solution culture was $Y = -2.1712\ (X) + 104.1922$ ($r^2 = 0.38$), while in sand culture, it was $Y = -4.2505\ (X) + 114.4129$ ($r^2 = 0.87$) and in soil culture the equation was $Y = -4.6805\ (X) + 106.7743$ ($r^2 = 0.86$), where X is salinity level and Y is seedling emergence.

Salinity levels in which the results for seedling emergence under solution or sand culture are typical representative of the results under soil culture were calculated. These values were computed where the two types of growing media recorded around 50% of seedling emergence (C_{50}). A significant positive correlation ($r = 0.81$) was observed between seedling emergence at EC_{iw} 22 dS m^{-1} in solution culture and EC_e 12.8 dS m^{-1} in soil culture. Similar correlation between seedling emergence at EC_{iw} 26 dS m^{-1} in solution culture with EC_e 12.8 dS m^{-1} in soil culture was recorded to be $r = 0.92$. Thus, using this method, a large number of genotypes can be tested for salinity tolerance in a short period of time.

52.2.2 Genetic Studies under Salt Stress

Genetic variability is the key to any crop improvement program, and the extent to which the desirable characters are heritable is also important. For improving yield

and yield component characters, information on their genetic variability and their interrelationships in different characters is necessary. Partitioning the genotypic correlation coefficients of yield components into direct and indirect effects may help estimate the actual contribution of an attribute and its influence through other characters. Sixty genotypes were evaluated for their adaptation under semiarid saline soil conditions [24]. High variability was recorded for secondary branches/plant, siliqua/plant, 1000-seed weight, seed yield/plant, and seed yield/siliqua. Further low variability was recorded for seeds/siliqua and primary branches per plant. Seeds/plant were least affected by salinity. Seeds bearing siliqua/plant and seed yield per plot showed high genetic coefficients of variation (GCV). Heritability was very high for the number of seeds/siliqua (99.74) and 1000-seed weight (61.44) and moderate for siliqua length (39.72). Genetic advance was high for seed-bearing siliqua/plant (15.02) and plant height (11.89), followed by seeds/siliqua (5.69) and sterile siliqua/plant (8.33). Genetic advance as percent of mean was high for seeds/siliqua (45.73) and 1000-seed weight (42.91), followed by seed yield per plant. High heritability associated with increased genetic advance indicated additive gene effects and greater possibility for improvement. Variability studies in 30 genotypes of Indian mustard indicated higher estimates of genetic and phenotypic coefficient of variation (PCV), heritability, and genetic advance for the number of primary branches and siliquae/plant on normal soil; and for seedling emergence, the number of siliquae on main shoot, the number of secondary branches/plant, and seed yield on saline soil. The number of siliquae/plant on normal soil and seedling emergence and plant height on saline soil had significant positive association with seed yield. Seedling emergence and plant height exhibited maximum direct, positive effects on seed yield on saline soil.

Furthermore, genetic parameters for variability were also studied under alkali stress conditions in 19 genotypes of Indian mustard [25]. The GCV, PCV, heritability, and genetic advance as percent of mean were high for 1000-seed weight on normal soil and for plant height, secondary branches per plant, the number of siliqua per main shoot length, and seed yield per plant on alkali soils. Correlation analysis revealed that seed yield per plant has positive and significant association for primary branches per plant and main shoot length on normal soil, and for plant height, secondary branches per plant and number of siliqua per main shoot length in alkali stress conditions. The path analysis showed that secondary branches per plant had maximum direct effect on seed yield under alkali stress conditions. These studies point out that the framing of selection criteria could be based on the number of primary branches per plant, main shoot length, and 1000-seed weight for normal conditions and secondary branches per plant, number of siliqua per main shoot length, and seed yield per plant under alkali stress conditions.

52.2.3 Salt-Tolerant Varieties

Among different *Brassica* species, Indian mustard (raya) showed higher tolerance to salinity/alkalinity stresses compared to *Gobhi sarson*, *Karan rai*, and yellow and brown

Table 52.1 Characteristics of Indian mustard varieties CS52, CS54, and CS56 released by CVRC for salt-affected soils of India.

Variety	CS52	CS54	CS56
Year of release	1997	2005	2008
Plant height	170 cm	160 cm	202 cm
Maturity duration	135 days	121 days	132 days
Grain type	Medium	Bold	Medium
1000-seed weight	4.0 g	5.3 g	4.4 g
Salinity tolerance	EC_e 6–9 dS m^{-1}	EC_e 6–9 dS m^{-1}	ECe 6–9 dS m^{-1}
Alkali tolerance	Upto pH 9.3	Upto pH 9.3	Upto pH 9.3
Average seed yield	15–16 q ha^{-1}	16–19 q ha^{-1}	16–18 q ha^{-1}
Recommended date of sowing	First fortnight of October	First fortnight of October	First fortnight of October (normal conditions) and upto 10th November (late sown)
Recommended for	Punjab, Haryana, and Rajasthan	Punjab, Haryana, and Rajasthan	Late-sown areas of the country (Punjab, Haryana, and Rajasthan)

sarson. Different genotypes of Indian mustard also showed differential tolerance to saline and alkali stresses. Breeding efforts following conventional breeding appraoches at CSSRI, Karnal, led to the development of high-yield salt-tolerant genotypes of Indian mustard, namely, CS52 in 1997, CS54 in 2005, and CS56 in 2008. These varieties were released by the Central Varietal Release Committee (CVRC), Government of India, for their cultivation in Punjab, Haryana, Rajasthan, and Gujarat States of India (Table 52.1).

The salt-tolerant variety CS52 yields 20% higher in salt-affected soils compared to the high-yield released varieties of Indian mustard at the national level. However, its maturity is longer by 1 week compared to the well-known high-yield released varieties of Indian mustard, that is, Varuna, Kranti, and Pusa Bold. Plant height of CS52 is around 170–180 cm. This variety is more tolerant to insects and pests' attack compared to other released varieties of Indian mustard. This variety accumulates and compartmentalizes the toxic ions in the root part, hence restricting the concentration of toxic ions in leaves and stem. Further, the toxic ions get accumulated in lower leaves, which are shed ultimately reducing the effects of toxic ions on the plant. These processes help the plant to survive better under salt stress conditions. Another characteristic of this variety is better adaptability under high temperature conditions during germination and seedling emergence stages.

These varieties can grow economically in saline soils up to a soil salinity level of EC_e 9 dS m^{-1} and in alkali soils up to pH 9.3. Within the salt-tolerant limits of this variety, CS52 and CS56 yield more than 1.6 ton ha^{-1}, whereas CS54 can yield upto 1.9 ton ha^{-1}. By growing in saline soils and even irrigating with saline waters, 39% oil content has been determined from the seeds of these varieties. The latest released variety CS56 has the additional advantage of delayed sowing upto 10th November without significant reduction in seed yield besides the salt tolerance characteristics.

52.3
"Omics" Approaches for Abiotic Stress Tolerance

Plant research has the potential to contribute significantly to solve several of the most daunting problems that our planet and we face in the twenty-first century. Developing crop plants with ability to tolerate abiotic stresses is need of the day that demands modern, novel strategies for a thorough understanding of plant's response to abiotic stresses. In particular, an array of innovative "omics" approaches such as genomics, proteomics, transcriptomics, and metabolomics are evolving at rapid pace, which is empowering the scientists to systematically analyze the genome at various levels and their effect on phenotypic variability. In contrast to the traditional approaches that mostly focus on one or a few genes at a time, omics tools particularly genomics allowed the use of important strategies such as genome-wide expression profiling that is useful to identify genes associated with stress response. Furthermore, proteomics helped a lot to analyze the relationship between gene expression (transcriptomics) and metabolism (metabolomics). Metabolomic studies, thus along with transcriptomics and proteomics, and their integration with systems biology, will lead to strategies to alter cellular metabolism for adaptation to abiotic stress conditions. Overall, the "omics" research approaches have produced copious data for living systems, which have necessitated the development of systems biology to integrate multidimensional biological information into networks and models. Applications of systems biology to plant science have been rapid and have increased our knowledge about circadian rhythms, multigenic traits, stress responses, and plant defenses, and have advanced the virtual plant project. In the proceeding pages we will discuss in detail the "omics" approaches such as genomics, proteomics, and metabolomics for abiotic stress tolerance in mustard.

52.3.1
Genomics Approaches and Abiotic Stress Tolerance in *B. juncea*

The application of genomics-type technologies is beginning to have an impact, enhancing our understanding of plant responses to abiotic stresses that interfere with the normal growth and metabolism of plants. The ongoing genome-sequencing project will give access to the required genes and will facilitate the analysis of their expression.

Abiotic stresses such as low temperature and high salinity adversely affect the seedling vigor and fertility of *B. juncea* and consequent reduction in yield [26]. Studies on *B. juncea* [27, 28] have demonstrated the coordinated transcriptional regulation of genes involved in sulfur transport and assimilation and in GSH biosynthesis. The Cd exposure, consequently, induces phytochelatin synthesis from glutathione (GSH). Zhu *et al.* [29, 30] overexpressed the γ-glutamylcysteine synthetase gene from *Escherichia coli* in *B. juncea*, resulting in increased biosynthesis of GSH and PCs and an increased tolerance to cadmium (Cd). A similar approach was taken with *Arabidopsis*; γ-glutamylcysteine synthetase was expressed in both sense and antisense orientations, resulting in plants with a wide range of GSH levels [31]. Flocco *et al.* [32]

tested the hypothesis that transgenic Indian mustard plants overexpressing ECS or GS, which have twofold levels of GSH and total nonprotein thiols, would have enhanced tolerance to organic pollutants, including the PAH phenanthrene. Exposure of plants to organic pollutants significantly enhanced total nonprotein thiol levels in both wild-type and transgenic plants [32]. Flocco *et al*. [32] concluded that GSH could be important for detoxification via conjugation to GSH, of many organic xenobiotics including phenanthrene, and that overexpression of enzymes involved in GSH biosynthesis offers a promising approach to create plants with the enhanced capacity to tolerate not only heavy metals but also certain organic pollutants. Besides the genes mentioned above, a range of other stress-related proteins have been employed in raising transgenic plants with improved tolerance toward various abiotic stresses as mentioned in Table 52.2.

52.3.2
Proteomic Approaches and Abiotic Stress Tolerance in *B. juncea*

The simple paradigm, gene to mRNA to protein, belies the complexity of the relationship between a stretch of genomic DNA and its cognate protein products. The transcriptome analyses of gene expression at the mRNA level have contributed greatly to our understanding of abiotic stress tolerance in plants. However, the level of mRNA does not always correlate well with the level of protein, the key player in the cell [33]. Therefore, it is insufficient to predict protein expression level from quantitative mRNA data. This is mainly due to posttranscriptional regulation mechanisms such as nuclear export and mRNA localization, transcript stability, translational regulation, and protein degradation. Proteome studies aim at the complete set of proteins encoded by the genome and thus complement the transcriptome studies. A proteome constitutes all different proteins in a particular "body." The body may be an organism, as in yeast proteomics [34], and can also be a subcellular particle such as an organelle [35, 36] or a protein complex [37].

52.3.3
Metabolomics Approaches and Abiotic Stress Tolerance in *B. juncea*

Undoubtedly, transcriptomic and proteomic data are important steps in deciphering a complex biological process, but they are still insufficient since most biological processes are ultimately mediated by cell metabolites. Metabolomics is considered to provide a direct "functional readout of the physiological state" of an organism. Besides, alternative mRNA splicing, protein turnover rates, and posttranslational modifications that modulate protein activity imply that changes in the transcriptome or proteome do not always correspond to alterations in the cell metabolome [38]. Target analysis, metabolite profiling, and metabolic fingerprinting are different conceptual approaches in metabolomics that can be used for a large range of applications, including phenotyping of genetically modified plants, substantial equivalence testing, determination of gene function, and monitoring responses to biotic and abiotic stress. Metabolomics can therefore be seen as bridging the gap

Table 52.2 Transgenic *Brassica* plants overexpressing genes of various pathways for stress tolerance.

Gene	Gene function	Gene source	Target *Brassica*	Phenotype	Reference
APS1	Plastidic ATP sulfurylase	*Arabidopsis thaliana*	*B. juncea*	Se tolerance	[40]
codA	Choline oxidase (glycine-betaine synthesis)	*Arthrobacter globiformis*	*B. juncea* cv. Pusa Jai Kisan	Choline tolerance	[41]
codA	Choline oxidase (glycine-betaine synthesis)	*A. globiformis*	*B. napus*	Salinity tolerance and enhance shoot growth	[42]
Gor	Glutathione reductase	*E. coli*	*B. juncea*	Increased GR activity	[43]
AtNHX1	Vacuolar Na^+/H^+ antiporter	*B. napus*	*B. napus*	Salt tolerance, growth, seed yield, and seed oil quality	[44]
SOD	Mn superoxide dismutase	*Triticum aestivum*	*B. napus*	Aluminum tolerance	[45]
WGA	Wheat germ agglutinin	*T. aestivum*	*B. juncea* cv. RLM-198	Resistance to *Lipaphis erysimi* Kalt.	[46]
CGS	Cystathionine synthase	*A. thaliana* (L.) Heynh	*B. juncea*	Selenium tolerance due to enhanced Se volatilization	[47]
CIT1	Mitochondrial citrate synthase	*A. thaliana*	*B. napus* cv. Westar	Increased root citrate exudation and Al tolerance	[48]
cpSL	Selenocysteine lyase	Mouse	*B. juncea*	Selenium tolerance	[49]
Parp1; Parp2	Poly(ADP-ribose) polymerase	*A. thaliana*	*B. juncea*	Silencing induces drought and heat tolerance	[50]
SMT	Selenocysteine methyltransferase	*Astragalus bisulcatus*	*B. juncea*	Selenium tolerance and accumulation	[51]
codA	Choline oxidase (glycine-betaine synthesis)	*A. globiformis*	*B. juncea*	Tolerance to stress-induced photoinhibition	[52]
SMT	Selenocysteine methyltransferase	*A. bisulcatus*	*B. juncea*	Selenium hyperaccumulation tolerance	[53]
PR10	Pathogenesis related	*Pisum sativum*	*B. napus*	Enhance germination and growth in the presence of NaCl	[54]
betA	Betaine aldehyde	*E. coli*	*B. oleracea*	Salinity tolerance	[55]

rolD	ACC deaminase	*Pseudomonas putida UW4*	*B. napus* var. *Westar*	Lower ethylene and nickel tolerance	[56]
ERA1	Farnesyltransferase	*A. thaliana*	*B. napus*	Antisense; promote drought resistance in the field	[57]
BNCBF5- and *17*	CBF/DREB1-like transcription factors	*B. napus* cv. *Jet neuf*	*B. napus*	Freezing tolerance and photosynthetic capacity	[58]
Lea	Group 3 late-embryogenesis abundant	*B. napus*	*B. campestris*	Salinity and drought tolerance	[59]
APS + SMT	ATP sulfurylase, selenocysteine methyltransferase	*A. bisulcatus*	*B. juncea*	Selenium tolerance and accumulation from selenate	[60]
Class I basic glucanase	Class I basic glucanase	Tomato	*B. juncea*	Resistance to *Alternaria brassicae*	[61]
PgNHX1	Na^+/H^+ antiporter	*Pennisetum glaucum*	*B. juncea*	Tolerance to high salinity	[62]
AtPCS1	Phytochelatin synthesis	*A. thaliana*	*B. juncea*	As and Cd tolerance	[63]
AtPCS1	Phytochelatin synthesis	*A. thaliana*	*B. juncea*	Cd and Zn tolerance	[64]
cpSL and SMT	Selenocysteine lyase and selenocysteine methyltransferase	*A. bisulcatus*	*B. juncea*	Enhanced potential for selenium phytoremediation under field conditions	[65]
BnPtdIns-PLC2	Phosphatidylinositol-specific phospholipase C	*B. napus*	*Canola*	Drought resistance, early flowering	[66]
ERA1	Farnesyltransferase	*A. thaliana*	*B. napus*	When downregulated promotes drought resistance	[67]
Th1PK2	Inositol polyphosphate kinase	*Thellungiella halophila*	*B. juncea*	Salt and drought resistance	[68]

between genotype and phenotype. Metabolic changes underpin plant development and responses to applied stresses, and that metabolic information reflects biological endpoints more accurately than transcript or protein analysis. Hence, the only way to the complete understanding of both gene function and molecular events controlling complex plant processes is to analyze the transcriptome, the proteome, and the metabolome in an integrative manner [39].

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References

1 Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.*, **444**, 139–158.
2 Khan, N.A. and Singh, S. (2008) *Plant Responses to Abiotic Stresses*, IK International, New Delhi.
3 Tuteja, N. (2007) Mechanisms of high salinity tolerance in plants. *Methoths Enzymol.*, **428**, 419–438.
4 Purty, R.S., Kumar, G., Singla-Pareek, S.L., and Pareek, A. (2008) Invited contribution towards salinity tolerance in *Brassica*: an overview. *Physiol. Mol. Biol. Plants*, **14**, 15–22.
5 Gill, S.S., Khan, N.A., Anjum, N.K., and Tuteja, N. (2011) Amelioration of cadmium stress in crop plants by nutrients management: morphological, physiological and biochemical aspects, in *Plant Nutrition and Abiotic Stress Tolerance III. Plant Stress 5 (Special Issue 1)* (eds N.A. Anjum and F. Lopez-Lauri), pp. 1–23.
6 Bray, E.A., Bailey-Serres, J., and Weretilnyk, E. (2000) Responses to abiotic stresses, in *Biochemistry and Molecular Biology of Plants* (eds W. Gruissem, B. Buchannan, and R. Jones), American Society of Plant Physiologists, Rockville, MD, pp. 1158–1249.
7 Nakashima, K., Ito, Y., and Shinozaki, K.Y. (2009) Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. *Plant Physiol.*, **149**, 88–95.
8 Kumar, G., Purty, R.S., Sharma, M.P., Singla-Pareek, S.L., and Pareek, A. (2009) Physiological responses among *Brassica* species under salinity stress show strong correlation with transcript abundance for SOS pathway-related genes. *J. Plant Physiol.*, **166**, 507–520.
9 Zhang, G.Q., Tang, G.X., and Zhou, W.J. (2003) A preliminary study on the interspecific hybridization of *B. compestris* and *B. oleracea* through ovary culture. *Sci. Agric. Sin.*, **36**, 1409–1413.
10 Damodaran, T. and Hegde, T.M. (2002) *Oilseeds Situation: A Statistical Compendium*, DOR, Hyderabad, pp. 210–229.
11 Khan, N.A., Singh, S., Nazar, R., and Lone, P.M. (2007) The source–sink relationship in mustard. *Asian Aust. J. Plant Sci. Biotechnol.*, **1**, 10–18.
12 Weiss, E.A. (1983) *Oilseed Crops*, Longman Inc, New York.
13 Chopra, V.L. and Prakash, S. (1991) Taxonomy, distribution and cytogenetics, in *Brassica Oilseed in Indian Agriculture* (eds V.L. Chopra and S. Prakash), Vikas Pub. House Pvt. Ltd., New Delhi, pp. 257–304.
14 Lionneton, E., Aubert, G., Ochatt, S., and Merah, O. (2004) Genetic analysis of agronomic and quality traits in mustard (*Brassica juncea*). *Theor. Appl. Genet.*, **109**, 792–799.

15 Oram, R.N., Kirk, J.T.O., Veness, P.E., Hurlstone, C.J., Edlington, J.P., and Halsall, D.M. (2005) Breeding Indian mustard [*Brassica juncea* (L.) Czern.] for cold-pressed, edible oil production; a review. *Aust. J. Agric. Res.*, **56**, 581–596.

16 Renard, S. and Mcgregor, S. (1992) Antithrombogenic effect of erucic acid poor rapeseed oil in the rats. *Rev. Fr. Crop Cros.*, **23**, 393–396.

17 Dhillon, S.S., Kumar, P.R., and Gupta, N. (1992) Breeding objectives and methodology, in *Breeding Oilseed Brassicas* (eds K.S. Labana, S.S. Banga, and S.K. Banga), Narosa Publishing House, New Delhi, pp. 10–17.

18 Gómez-Campo, C. and Tortosa, M.E. (1974) The taxonomic and evolutionary significance of some juvenile characters in the tribe Brassiceae (Cruciferae). *Bot. J. Linn. Soc.*, **69**, 105–112.

19 Prakash, S. (1980) Cruciferous oilseeds in India, in *Brassica Crops and Wild Allies: Biology and Breeding* (eds S. Tsunoda, K., Hinata and C. Gomez-Campo), Japan Science Society Press, Tokyo, pp. 151–163.

20 Hemingway, J.S. (1976) Mustards, in *Evolution of Crop Plants* (eds N.W. Simmonds and J. Smart), Longman, London, pp. 82–86.

21 UN (1935) Genomic-analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Jpn. J. Bot.*, **7**, 389–452.

22 Sharma, P.C. (2003) Salt tolerance of Indian mustard (*Brassica juncea* L.): factors affecting growth and yield. *Indian J. Plant Physiol.*, **8** (Special issue (NS)), 368–372.

23 Sinha, T.S., Singh, D., Sharma, P.C., and Sharma, H.B. (2003) Rapid screening methodology for salt tolerance during germination and seedling emergence in Indian mustard (*Brassica juncea* L.). *J. Plant Physiol.*, **8** (Special issue (NS)), 363–367.

24 Sinha, T.S. (1991) Genetic adaptation of Indian mustard (*Brassica juncea*) to semi-arid saline soil conditions. *Ind. J. Agric. Sci.*, **61**, 251–254.

25 Sinha, T.S., Singh, D., Sharma, P.C., and Sharma, H.B. (2002) Genetic variability, correlation and path coefficient studies and their implications of selections of high yielding genotypes in Indian mustard (*Brassica juncea* L) under normal and sodic soil conditions. *Ind. J. Coastal Agric. Res.*, **20**, 31–36.

26 Zhang, Y.X., Xu, J., Han, L., Wei, W., Guan, Z.Q., Cong, L., and Chai, T.Y. (2006) Highly efficient shoot regeneration and *Agrobacterium*-mediated transformation protocol of *Brassica juncea*. *Plant Mol. Biol. Rep.*, **24**, 255–255.

27 Schäfer, H.J., Haag-Kerwer, A., and Rausch, T. (1998) cDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy metal accumulator *Brassica juncea* L: evidence for Cd-induction of a putative mitochondrial γ-glutamylcysteine synthetase isoform. *Plant Mol. Biol.*, **37**, 87–97.

28 Lee, S. and Leustek, T. (1999) The effect of cadmium on sulfate assimilation enzymes in *Brassica juncea*. *Plant Sci.*, **141**, 201–207.

29 Zhu, O.Y.L., Pilon-Smits, E.A.H., Jouanin, L., and Terry, N. (1999) Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. *Plant Physiol.*, **119**, 73–79.

30 Zhu, Y.L., Pilon-Smits, E.A.H., Jouanin, L., and Terry, N. (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. *Plant Physiol.*, **121**, 1169–1177.

31 Xiang, C., Werner, B.L., Christensen, E.M., and Oliver, D.J. (2001) The biological functions of glutathione revisited in *Arabidopsis* transgenic plants with altered glutathione levels. *Plant Physiol.*, **126**, 564–574.

32 Flocco, C.G., Lindblom, S.D., and Pilon-Smits, E.A.H. (2004) Overexpression of enzymes involved in glutathione synthesis enhances tolerance to organic pollutants in *Brassica juncea*. *Int. J. Phyto.*, **6**, 289–304.

33 Yan, S.P., Zhang, Q.Y., Tang, Z.C., Su, W.A., and Sun, W.N. (2006) Comparative proteomic analysis provides new insights into chilling stress responses in rice. *Mol. Cell Proteom.*, **5**, 484–496.

34 Washburn, M.P., Wolters, D., and Yates, J.R., 3rd (2001) Large-scale analysis of the

yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.*, **2001**, **19**, 242–247.

35 Prime, T.A., Sherrier, D.J., Mahon, P., Packman, L.C., and Dupree, P. (2000) A proteomic analysis of organelles from *Arabidopsis thaliana*. *Electrophoresis*, **21**, 3488–3499.

36 Peltier, J.B., Friso, G., Kalume, D.E., Roepstorff, P., Nilsson, F., Adamska, I., and van Wijk, K.J. (2000) Proteomics of the chloroplast: systematic identification and targeting analysis of lumenal and peripheral thylakoid proteins. *Plant Cell*, **12**, 319–341.

37 Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R.J. (2000) Proteasome proteomics: identification of nucleotide sensitive proteasome-interacting proteins, by mass spectrometric analysis of affinity-purified proteasomes. *Mol. Biol. Cell*, **11**, 3425–3439.

38 Sumner, L.W., Mendes, P., and Dixon, R.A. (2003) Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochem.*, **62**, 817–836.

39 Dixon, R.A. (2001) Natural products and plant disease resistance. *Nature*, **411**, 843–847.

40 Pilon-Smits, E.A.H., Hwang, S., Lytle, C.M., Zhu, Y., Tai, J.C., Bravo, R.C., Chen, Y., Leustek, T., and Terry, N. (1999) Overexpression of ATP sulfurylase in Indian mustard leads to increased selenate uptake, reduction, and tolerance. *Plant Physiol.*, **119**, 123–132.

41 Prasad, K.V.S.K., Sharmila, P., Kumar, P.A., and Pardha Saradhi, P. (2000) Transformation of *Brassica juncea* (L.) Czern with bacterial codA gene enhances its tolerance to salt stress. *Mol. Breed.*, **6**, 489–499.

42 Huang, J., Hirji, R., Adam, L., Rozwadowski, K.L., Hammerlindl, J.K., Keller, W.A., and Selvaraj, G. (2000) Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiol.*, **122**, 747–756.

43 Pilon-Smits, E.A., Zhu, Y.L., Sears, T., and Terry, N. (2000) Overexpression of glutathion reductase in *Brassica juncea*: effects on cadmium accumulation and tolerance. *Physiol. Plant.*, **110**, 455–460.

44 Zhang, H.X., Hodson, J.N., Williams, J.P., and Blumwald, E. (2001) Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci. USA*, **98**, 12832–12836.

45 Basu, U., Good, A.G., and Taylor, G.J. (2001) Transgenic *Brassica napus* plants overexpressing aluminium-induced mitochondrial manganese superoxide dismutase cDNA are resistant to aluminium. *Plant Cell Environ.*, **24**, 1269–1278.

46 Kanrar, S., Venkateswari, J., Kirti, P.B., and Chopra, V.L. (2002) Transgenic Indian mustard (*Brassica juncea*) with resistance to the mustard aphid (*Lipaphis erysimi* Kalt.). *Plant Cell Rep.*, **20**, 976–981.

47 Van Huysen, T., Abdel-Ghany, S., Hale, K.L., LeDuc, D., Terry, N., and Pilon-Smits, E.A.H. (2003) Overexpression of cystathionine-synthase enhances selenium volatilization in *Brassica juncea*. *Planta*, **218**, 71–78.

48 Anoop, V.M., Basu, U., McCammon, M.T., McAlister-Henn, L., and Taylor, G.J. (2003) Modulation of citrate metabolism alters aluminum tolerance in yeast and transgenic canola overexpressing a mitochondrial citrate synthase. *Plant Physiol.*, **132**, 2205–2217.

49 Garifullina, G.F., Owen, J.D., Lindblom, S.D., Tufan, H., Pilon, M., and Pilon-Smit, E.A.H. (2003) Expression of a mouse selenocysteine lyase in *Brassica juncea* chloroplasts affects selenium tolerance and accumulation. *Physiol. Plant.*, **118**, 538–544.

50 Block, M.D., Christoph, V., De, B.D., and Marc, C. (2005) Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. *Plant J.*, **41**, 95–106.

51 LeDuc, D.L., Tarun, A.S., Montes-Bayon, M., Meija, J., Malit, M.F., Wu, C.P., AbdelSamie, M., Chiang, C.Y., Tagmount, A., deSouza, M., Neuhierl, B., Bock, A., Caruso, J., and Terry, N. (2004) Overexpression of selenocysteine methyltransferase in *Arabidopsis* and

Indian mustard increases selenium tolerance and accumulation. *Plant Physiol.*, **135**, 377–383.

52 Prasad, K.V.S.K. and Pardha Saradhi, P. (2004) Enhanced tolerance to photoinhibition in transgenic plants through targeting of glycinebetaine biosynthesis into the chloroplasts. *Plant Sci.*, **166**, 1197–1212.

53 Danika, L.L.D., Tarun, A.S., Montes-Bayon, M., Meija, J., Malit, M.F., Wu, C.P., AbdelSamie, M., Chiang, C.Y., Tagmount, A., deSouza, M., Neuhierl, B., Bock, A., Caruso, J., and Terry, N. (2004) Overexpression of selenocysteine methyltransferase in *Arabidopsis* and Indian mustard increases selenium tolerance and accumulation. *Plant Physiol.*, **135**, 377–383.

54 Srivastava, S., Fristensky, B., and Kav, N.N.V. (2004) Constitutive expression of a PR10 protein enhances the germination of *Brassica napus* under saline conditions. *Plant Cell Physiol.*, **45**, 1320–1324.

55 Bhattacharya, R.C., Maheswari, M., Dineshkumar, V., Kirti, P.B., Bhat, S.R., and Chopra, V.L. (2004) Transformation of *Brassica oleracea* var. *capitata* with bacterial betA gene enhances tolerance to salt stress. *Sci. Horticul.*, **100**, 215–227.

56 Stearns, J.C., Shah, S., Greenberg, B.M., Dixon, D.G., and Glick, B.R. (2005) Tolerance of transgenic canola expressing 1-aminocyclopropane-1-carboxylic acid deaminase to growth inhibition by nickel. *Plant Physiol. Biochem.*, **43**, 701–708.

57 Yang, W., Jifeng, Y., Monika, K., Maryse, C., Angela, S., Charlene, M., Tina, U., Carlene, S., Jiangxin, W., David, D.T., Peter, M., and Huang, Y. (2005) Molecular tailoring of farnesylation for plant drought tolerance and yield protection. *Plant J.*, **43**, 413–424.

58 Savitch, L.V., Allard, G., Seki, M., Robert, L.S., Tinker, N.A., Huner, N.P.A., Shinozaki, K., and Singh, J. (2005) The effect of overexpression of two brassica CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. *Plant Cell Physiol.*, **46**, 1525–1539.

59 Park, B-.J., Liu, Z., Kanno, A., Kameya T. (2005) Increased tolerance to salt- and water-deficit stress in transgenic lettuce (*Lactuca sativa* L.) by constitutive expression of LEA. *Plant Growth Regul.*, **45**, 165–171.

60 LeDuc, D.L., Abdelsamie, M., Móntes-Bayon, M., Wu, C.P., Reisingerand, S.J., and Terry, N. (2006) Overexpressing both ATP sulfurylase and selenocysteine methyltransferase enhances selenium phytoremediation traits in Indian mustard. *Environ. Pollut.*, **144**, 70–77.

61 Mondal, K.K., Bhattacharya, R.C., Koundal, K.R., and Chatterjee, S.C. (2007) Transgenic Indian mustard (*Brassica juncea*) expressing tomato glucanase leads to arrested growth of *Alternaria brassicae*. *Plant Cell Rep.*, **26**, 247–252.

62 Rajagopal, D., Agarwal, P., Tyagi, W., Singla-Pareek, S.L., Reddy, M.K., and Sopory, S.K. (2007) *Pennisetum glaucum* Na^+/H^+ antiporter confers high level of salinity tolerance in transgenic *Brassica juncea*. *Mol. Breed.*, **19**, 137–151.

63 Gasic, K. and Korban, S.S. (2007) Transgenic Indian mustard (*Brassica juncea*) plants expressing an *Arabidopsis* phytochelatin synthase (AtPCS1) exhibit enhanced As and Cd tolerance. *Plant Mol. Biol.*, **64**, 361–369.

64 Gasic, K. and Korban, S.S. (2007) Transgenic Indian mustard (*Brassica juncea*) plants expressing an *Arabidopsis* phytochelatin synthase (AtPCS1) exhibit enhanced As and Cd tolerance. *Plant Mol. Biol.*, **64**, 361–369.

65 Banuelos, G., LeDuc, D.L., Pilon-Smits, E.A.H., and Terry, N. (2007) Transgenic Indian mustard overexpressing selenocysteine lyase or selenocysteine methyltransferase exhibit enhanced potential for selenium phytoremediation under field conditions. *Environ. Sci. Technol.*, **41**, 599–605.

66 Georges, F., Das, S., Ray, H., Bock, C., Nokhrina, K., Kolla, V.A., and Keller, W. (2009) Over-expression of *Brassica napus* phosphatidylinositol-phospholipase C2 in canola induces significant changes in gene expression and phytohormone distribution patterns, enhances drought tolerance and promotes early flowering and maturation. *Plant Cell Environ.*, **32**, 1664–1681.

67 Wang, Y., Beaith, M., Chalifoux, M., Ying, J., Uchacz, T., Sarvas, C., Griffiths, R.,

Kuzma, M., Wan, J., and Huang, Y. (2009) Shoot-specific down-regulation of protein farnesyltransferase (-subunit) for yield protection against drought in canola. *Mol. Plant*, **2**, 191–209.

68 Zhu, J.Q., Zhang, J.T., Tang, R.J., Lv, Q.D., Wang, Q.Q., Yang, L., and Zhang, H.X. (2009) Molecular characterization of ThIPK2, an inositol polyphosphate kinase gene homolog from *Thellungiella halophila*, and its heterologous expression to improve abiotic stress tolerance in *Brassica napus*. *Physiol. Plant.*, **136**, 407–425.

Section IIIE Other Crops

Improving Crop Resistance to Abiotic Stress, First Edition.
Edited by Narendra Tuteja, Sarvajeet Singh Gill, Antonio F. Tiburcio, and Renu Tuteja
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53
Cotton: Genetic Improvement for Drought Stress Tolerance – Current Status and Research Needs

E. AbouKheir, M.S. Sheshshayee, T.G. Prasad, and Makarala Udayakumar

A significant part of cotton production comes from water-limited rain-fed areas. Being a commercially important crop, improvement of drought tolerance has received increasing attention in crop improvement programs. The marked genetic variability in desirable traits among cotton germplasm has great potential for exploitation through conventional and/or molecular breeding approaches. While most research activities targeted the cotton species *Gossypium hirsutum*, very limited physiological information is available in other cotton species. At present, the emphasis is to identify and assess variability in specific traits that have relevance in enhancing drought stress tolerance. Ensuing global research has enumerated a few traits such as roots, water use efficiency, and cellular-level tolerance as the most important besides several other physiological traits. Being quantitatively inherited, numerous research attempts have been initiated to identify the relevant QTL and to pyramid them on to superior genetic backgrounds. Likewise, phenomenal progress has also been made in understanding the molecular regulation of drought tolerance, and to identify the functional/regulatory genes for development of drought-tolerant transgenics in various crops. Such approaches are minimal in cotton and it is imperative that such approaches have great promise to develop genotypes with agronomically desirable traits through molecular breeding and/or transgenics to improve cotton yield and fiber quality. Hence, research priority should be laid on utilization of the available genomic resources through effective integration of conventional breeding with molecular breeding tools. Furthermore, cotton species, namely, *G. arboreum* and *G. herbaceum* need to be explored to assess the variability in stress-adaptive traits. This chapter examines the cotton drought response and provides an insight into the relevant traits that have a role in enhancing drought tolerance. In addition, the prospects of conventional crop improvement approaches vis-a-vis molecular breeding and transgenic approaches that have relevance to achieving crop drought adaptation without sacrificing the yield and quality. It also provides insight into the research needs on the quantitative and association genetics with an ecophysiological understanding of the cotton genomic resources and to better inform crop improvement program.

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53.1 Introduction

Cotton belongs to Malvaceae family and tribe *Gossypieae* that includes eight genera [1, 2] with four of these genera (*Lebronnecia*, *Cephalohibiscus*, *Gossypioides*, and *Kokia*) having restricted geographic distributions [1, 2]. The genera *Hampea*, *Cienfuegosia*, *Thespesia*, and *Gossypium* are moderately larger genera with broader geographic range. Fiber cotton belongs to *Gossypium*, which is the largest and most widely distributed genus in *Gossypieae* tribe with approximately 50 species [3] grown in arid to semiarid regions of the tropic and subtropic regions [3–5]. Of them, only two species, *Gossypium arboreum* and *G. herbaceum*, are commercially cultivated and known collectively as "Desi" cottons, and are the Asiatic or Old World short-staple cotton. Owing to their ability to withstand drought stress, these diploid species are generally cultivated in marginal, drought-prone environments of Asia. These diploid Desi cottons are also known for their ability to resist sucking pests such as hoppers, white flies, thrips, and aphids, and leaf curl virus. However, owing to their short, coarse, and weak fiber and undesirable boll and plant features, the Desi cottons were gradually displaced by *G. hirsutum* in Asia.

Evolutionarily, *G. hirsutum* has seven landraces, namely, *palmeri*, *morilli*, *richmondii*, *yucatenanse*, *punctatum*, *marie galante*, and *latifolium*. The race *latifolium* is widely cultivated in the world and *yucatannse* appears to be the wild progenitor of upland cotton [6]. *G. barbadense* is the other domesticated allotetraploid that yields extralong staples or extrafine quality cotton fiber. This species shares about 3% of the total world cotton market [7]. The exploitation of the genetic variability of these species is yet to be realized using modern biological tools in conjunction with conventional approaches.

Most cotton fiber production comes from species *G. hirsutum* and *G. barbadense*, with a minor contribution from *G. arboreum* and *G. herbaceum*. Although cotton is cultivated in more than 70 countries [8] ranging from tropical to subtropical regions with more than 350 million ha [9], almost 65% of global area is in four countries India, China, the United States of America, and Pakistan (Table 53.1). India ranks first in terms of cultivation area, occupying 26% of the world cotton area, followed by China, the United States, and Pakistan. Globally, 115 million bales were produced during 2006–2007 season. China contributes 24% of total production followed by India, the United States, and Pakistan. Although Australia ranks first in productivity (1887 kg ha^{-1}), it contributes to only 2% of total cotton production with less than 1% of global cotton area (Table 53.1). However, among the major cotton growing countries, China holds the highest productivity (1130 kg ha^{-1}) followed by the United States and Pakistan with productivity of 858 and 703 kg ha^{-1}, respectively. India with a yield of 494 kg ha^{-1} is the lowest.

Major area of cotton cultivation in India is under rain-fed conditions, which occupy about 50%. The cultivated area is concentrated in the central part of India occupying about 66.66%, with only 23% under irrigated conditions followed by Southern India (40%) [10]. Several abiotic stresses such as temperature, soil ion concentrations,

Table 53.1 Major cotton-growing countries of the world.

Country	Area ('000 ha)	Production ('000 bales)	Yield (kg ha^{-1})
USA	5186	20 431	858
China	5300	27 500	1130
India	9250	21 000	494
Pakistan	3250	10 500	703
Australia	300	2600	1887
World	35175	115 590	715

Source: FAO [11].

light, and so on adversely affect cotton growth and development. However, the most devastating abiotic stress is soil moisture deficit and about 45% of the world's arable land suffers from chronically inadequate supplies of water for agriculture [12] including cotton [13]. It is projected that burgeoning human population and urbanization will further aggravate the increasing demand for fresh water both for human consumption and for agriculture. Furthermore, erratic and often insufficient rainfall witnessed in the recent past due to anthropogenic changes in the climate might further reduce the availability of fresh water. Although water and soil management practices have a significant role in sustaining productivity under water-limited conditions, a genetic enhancement of cotton genotypes with increased water use efficiency (WUE) as a seed-based technology has greater acceptability.

Despite increased interest to exploit the genomic resources for the development of drought-tolerant crops, it has been hindered by lack of knowledge of precise physiological characteristics that form the index diagnostic parameter of genetic variability and potential for improved productivity and quality under water-limited conditions. Using genetic mapping to dissect the inheritance of different complex traits is a powerful tool to distinguish heritability from casual associations between such traits [14]. In principle, it permits a direct analysis of the role of specific physiological traits in determining genetic variability and potential for productivity and quality parameters under abiotic stress conditions.

Cotton (*Gossypium* spp.) is a potential taxon to study the variability in genomic resources with potential for adaptation to water-limited conditions. Cotton originates from wild perennial plants adapted to semiarid, subtropical environments that experience periodic drought stress conditions. However, the modern cotton cultivars are the result of intensive selection to improve the yield of lint and its quality. Such a traditional selection with emphasis on maximal productivity under high-input and irrigated conditions has inadvertently eroded the genetic variability in drought tolerance [15]. However, considerable variation persists within and between the two cultivated tetraploid cotton species, *G. hirsutum* and *G. barbadense*, in physiological traits, which can be still exploitable.

53.2
Response of Cotton to Drought Stress

Water deficit stress has profound effects on plant growth, development, and quality. The first effect of the moisture stress may well be loss of turgor that affects the rate of cell expansion and ultimate cell size. The result is a decrease in growth rate, stem elongation, leaf expansion, and stomatal aperture with an overall decrease in the performance of the plant.

Such reduction in leaf area index (LAI) results in a reduced crop growth rate (CGR) under conditions of lower water supply [16–18]. Similar inferences where moisture stress during vegetative stage causes reduced leaf growth and leaf area have also been reported [19]. Smaller leaves, increased leaf senescence and abscission [20–22], lesser leaf numbers [23], reduced leaf area index at maturity and interception of less light by the crop, lesser plant height [19], and lower shoot-to-root ratio [19, 24] eventually decrease total dry matter produced by plant.

In one of our experiments [25], three upland cotton genotypes grown in two different water regimes (100 and 55% field capacity) showed significant reduction in leaf area (Figure 53.1a) and total dry matter (Figure 53.1b). This reduction in growth rates often corresponds linearly with water used by the crop [26]. A linear decrease in stem elongation has been reported as leaf water potential (LWP) declined and no further growth was observed when LWP was as low as −2.5 MPa [27–29].

These results clearly indicate that maintenance of leaf tissue water relations is most crucial to sustain growth rates under water-limited conditions. Therefore, greater emphasis needs to be laid on assessing the variability in root traits and the root growth response to drought stress. Significant changes in root growth patterns have been noticed during a drying cycle [30]. Stimulation of root proliferation is significantly greater as soil drying continues, a trend not observed in well-watered plants. Furthermore, during drying cycles, root growth appears closely associated with shoot growth, with elongation ceasing concurrently with cessation of growth in the shoot [31] and root elongation ceased when the soil water potential declined to less

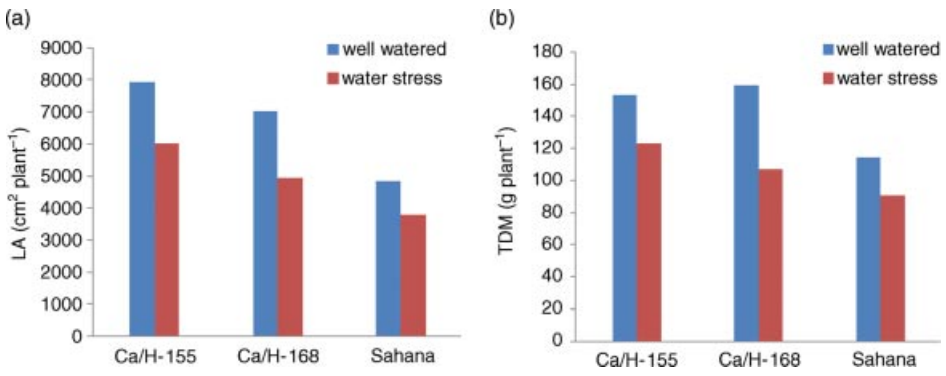

Figure 53.1 Effect of moisture stress on (a) leaf area and (b) total dry matter among three upland cotton genotypes grown into two different water regimes [25].

than −0.1 MPa. The taproot length was greater but not the root dry weight under moisture stress compared to control condition [19]. The leaf expansion was more sensitive to water stress than root elongation [32, 33].

Water deficit stress also affects boll production and thus cotton yield [34–36]. The abrupt cessation in boll formation depends on imbalance between plants photosynthetic capacity and assimilate demand [37, 38]. Moisture stress occurrence during the first 14 days after anthesis leads to boll abscission [39], but occurrence of stress after that period generally does not cause abscission [40]. However, stress later during boll formation adversely affects fiber quality. Fiber elongation was decreased when drought stress occurred during the fiber elongation stage [41–43] and if stress severity increased beyond the fiber elongation period, led to fiber immaturity and low micronaire [44]. On the contrary, mild drought stress can be beneficial due to increase in lint percentage over irrigated cotton [45] and occasionally increased micronaire [46]. Krieg [23] summarized the drought stress effects on cotton growth and productivity and demonstrated that the timing and severity of water deficit stress has a large effect on yield. Levi *et al.* [47] showed 31% reduction in seed cotton yield under moisture stress.

Soil water deficit causes a progressive and significant reduction in evapotranspiration (ET) with the extent of reduction dependant on the severity and duration of stress [27]. This reduced transpiration would lead to an increase in the canopy temperature predominantly due to a stress-induced stomatal closure [22]. The reduction in transpiration can also be attributed to a stress-induced reduction in leaf expansion [48–50], stomatal closure [51–53], and reduction in leaf metabolic activities leading to increased senescence. In cotton, photosynthesis is more sensitive to low water potential than the stomatal conductance [54, 55] mainly due to decrease in the synthesis and activity of photosynthetic enzymes under moisture stress conditions [56].

53.3
Trait-Based Breeding to Improve Tolerance to Abiotic Stress

Considering the economic importance of cotton and that a significant area of cotton production comes from water-limited rain-fed conditions, attempts to sustain or even increase yield potentials under stress is a major challenge. Through crop improvement efforts for higher absolute yields, further success in such conventional breeding may not provide the requisite yield advantage because of a narrow variability in yield among the improved cultivars [57, 58]. Therefore, understanding the plant physiological characteristics underlying yield formation can direct breeding both for specific integral traits and for crop response to stress leading to increased yield and cotton fiber quality.

Drought stress is a very complex stress, and both stress response and crop adaptation to stress are equally complicated. For achieving a comprehensive improvement in drought tolerance coupled with improved productivity under water-limited conditions, it appears that several diverse traits need to be introgressed into a single elite genetic background. Among a large number of adaptive and constitutive traits

that have been identified, traits associated with tissue–water relations need to be improved [59–61]. Traits associated with mining water from deeper soil layers (roots), water use efficiency [62, 63], and high-degree of cellular level tolerance [64] deserve greatest emphasis. Toward a trait-based breeding strategy, the first step is to systematically characterize the available cotton germplasm accessions for the variability in these traits and identify superior trait donor lines. In addition, it is also extremely important to assess variability in several morphophysiological traits such as the canopy architecture and leaf expansion traits.

53.3.1 Canopy Characters

Manipulating the canopy architecture through leaf characters can be a useful strategy in breeding for drought tolerance. Other traits such as early maturity [65, 66], reduced leaf area index, and higher canopy CO_2 uptake [67], high light-saturated single-leaf photosynthetic rates [68], a shorter sympodial plastochron [69], and increased numbers of flowers per season [70] also deserve exploitation. Meredith [71] found that a copy with subokra-type leaves yielded greater than okra, superokra, and normal leaf types. Stiller *et al.* [72] confirmed that cotton with the okra leaf morphology was more suitable for improved yield under dry land conditions.

53.3.2 Root Traits

Cotton root consists of a primary or "tap" root from which branch the secondary and tertiary roots [73]. The cotton root, whether primary or branch root, has a single layer of epidermal cells surrounding the root cortex. The endodermis, single-cell layer, surrounds the stele that contains the xylem and phloem vascular elements and a cell layer called the pericycle. The xylem elements are arranged either in a tetrarch (four distinct xylem bundles, the most common) or in a greater bundle arrangement [74]. The difference in arrangement is apparently genetically controlled and may result in significant genetic diversity in root characteristics.

The depth of root penetration depends on a number of factors such as soil moisture, structure, pH, temperature, and so on, but in general the taproot can reach depths of over 3 m and can elongate at a rate from less than 1 to over 6 cm per day. The elongation rate of the lateral or secondary roots would generally fall within the same range [75]. In general, the root system continues to grow and increase in length until young bolls begin to form [31] at which time root length declines as older roots die. New roots continue to be formed past this point, but the net result is a decline in total length [76].

A number of different root traits and morphophysiological traits have been implicated as important mechanisms that impart drought tolerance in cotton. These include distance from transition zone to the first main lateral root, taproot weight, number of lateral roots, seedling vigor, rapidity of root system development, and root-to-shoot ratio [24] and longer taproot length [19].

Several of the root-related traits show significant response to stress. An increase in root density in the soil layer between 70 and 180 cm was reported in cotton in dry soil profile compared to well-watered condition [30]. Pace *et al.* [19] found that under drought stress, there was increase in taproot length without any change in cotton taproot dry weight. Detrimental effects of drought on root growth were observed only after recovery where both taproot' and secondary roots' dry weight decreased [19, 33, 77]. Thus, increase in taproot length, at the expense of thickening, in response to drought may be a common response in cotton. Such a response may have evolved to permit cotton plants to survive drought by accessing water from deeper soil profiles [78]. Contrasting evidence exists that drought stress reduces cotton root growth, root elongation, root length density, and root distribution [32, 79, 80]. However, such root growth reductions are normally noticed when the stress is severe or when it is prolonged.

Although superior drought tolerance is associated with enhanced root growth, rapid root water uptake, maintenance of root viability at the soil surface, and rapid root regeneration after rewatering [81], no serious breeding efforts have been initiated till date to improve root traits [82]. Lack of appropriate phenotyping strategy for root traits is perhaps the most important constraint. Several techniques have been developed to study root parameters such as hydroponics [83, 84], minirhizotrons [85], minilysimeters [86], and tubes [87]. More recently, sophisticated imaging techniques have been developed for assessing root growth and development. Although these techniques have been used widely to measure root parameters, they suffer either from cumbersomeness of procedure or inability to screen large number of accessions. In addition, some of the techniques such as tubes, minilysimeters, and so on do not represent the correct phenotypic expression of roots, as they do not experience interplant competitions [88]. Similarly, hydroponic techniques also cannot be adopted to assess root growth variations over extended crop growth periods.

Most of these disadvantages can be overcome by growing plants in specially constructed "field root phenotyping structures" [88]. Briefly, these root structures are constructed using bricks and are raised beds 5 feet above ground and are 10 feet wide. The length of the structures can be varied based on the crop species and the number of accessions to be studied (Figure 53.2). Soil of the required type can be filled in these structures and plants are raised with the recommended spacing between plants. Thus, in such field root phenotyping structures plants are raised under near-natural conditions that experience interplant competition and hence would represent the best phenotypic assessment while determining the genetic variability [64, 88].

In a field study done at our center, 158 upland cotton germplasm accessions were screened for variability in root traits by raising plants in field root phenotyping structures (Figure 53.2). A significant genotypic variability in root length, root dry weight, root volume, and root-to-shoot ratio was observed [64]. Each of these parameters displayed a normal distribution illustrating the polygenic regulation of root traits (Figure 53.3).

Similarly, Basal *et al.* [89] reported a considerable genetic variation in root length, lateral root number, root fresh weight, lateral root dry weight, and total root dry weight of 68 cotton landraces. On the contrary, Roark and Quisenberry [90] observed low genetic variability in cotton cultivars. As most of the cultivars examined by them were

Figure 53.2 Specially constructed field root phenotyping structure to assess genetic variability in root traits in a large number of accessions [64].

selected from humid and high-rainfall conditions, the genotypes did not exhibit greater variability in root traits [90, 91].

Considering the importance of root traits for drought tolerance, scientists have constantly been looking toward evolving newer and more robust strategies for the assessment of genetic variability in root traits.

53.3.3
Oxygen Isotope Ratio as a Surrogate for Root Traits

Although the theory explaining the phenomenon of oxygen isotopic enrichment during evaporation of water from ocean surface is well known for almost four

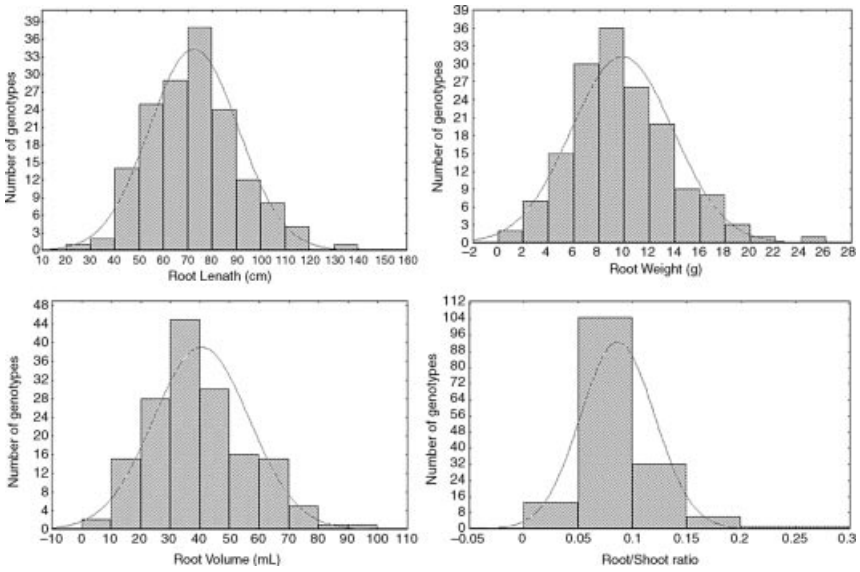

Figure 53.3 Significant genotypic variability observed for various root traits among cotton germplasm accessions [64].

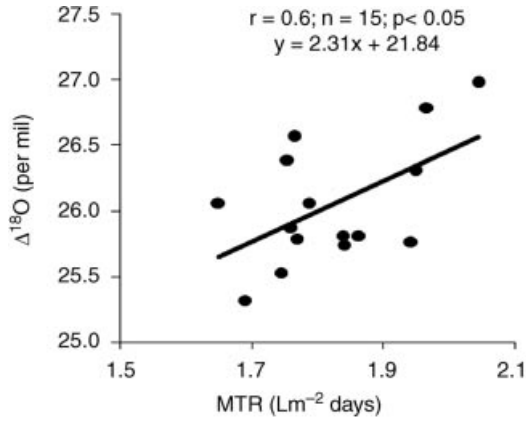

Figure 53.4 Relationship between oxygen isotope enrichment ($\Delta^{18}O$) and mean transpiration rate (MTR) among 15 cotton lines (*G. hirsutum*).

decades [92], the application of this theory to predict differences in transpiration rate has been fairly recent [93–95]. Furthermore, the relationship between stomatal conductance and leaf water ^{18}O enrichment has remained equivocal [96], though increased transpiration has been clearly shown to enrich leaf water ^{18}O [97, 98]. Sheshshayee *et al.* [99] and AbouKheir *et al.* [100] have provided experimental evidences and demonstrated that oxygen isotope enrichment is a powerful time-averaged surrogate for transpiration rate (Figure 53.4). Total transpiration is a function of canopy leaf area and the transpiration rate per unit leaf area. Accordingly, a strong positive relationship between the measured total transpiration and the predicted transpiration based on oxygen isotope enrichment was noticed among the 15 contrasting cotton lines (Figure 53.5a). Furthermore, total transpiration at a given leaf area depends on the ability of the root system in meeting the evaporative demand of the canopy. We observed a strong correlation between root biomass and the total transpiration predicted using oxygen isotope ratio (Figure 53.5b). These results clearly demonstrate the relevance of oxygen isotope enrichment as a surrogate for root traits.

53.3.4
Water Use Efficiency

Water use efficiency, the amount of biomass produced for unit volume of water transpired, is a key parameter that needs to be improved to produce better yield for a given amount of water or for having a stable yield with less water used. WUE can be considered part of resource management under moderate drought stress. Several researchers are of the opinion that there is only a small exploitable variability in WUE [100–102]. However, more recent studies have clearly demonstrated a significant genetic variability in WUE in several crop species [103–110] including cotton [100, 111–113].

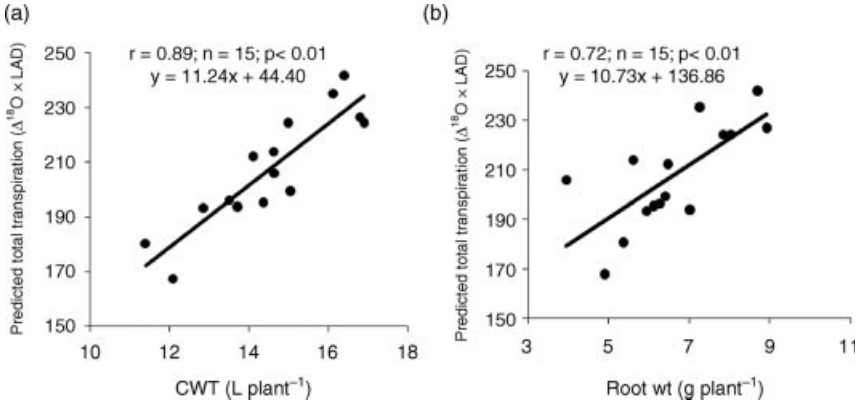

Figure 53.5 Relationship between predicted total water transpired ($\Delta^{18}O \times LAD$) with cumulative water transpired (a) and root biomass (g Plant^{-1}) (b) among 15 cotton lines (*G. hirsutum*) [100].

WUE can be measured at single-leaf level, as a ratio between the rate of carbon fixation by photosynthesis and the rate of transpiration, as a ratio of the photosynthesis rate and stomatal conductance since major water loss through the stomata [114–116]. WUE can also be measured at whole-plant level, the ratio between total dry matter accumulated during a season and amount of water transpired by plant [86]. Precise and reliable evaluation of WUE either at whole-plant level or at single-leaf level are complex, difficult to achieve, and expensive. A large drawback of WUE measured at whole-plant level is that it is cumbersome and hence cannot be used for large-scale screening. On the other hand, WUE measurement at single-leaf level is an instantaneous measurement, which fails to incorporate seasonal effects of WUE [113]. Measures of WUE that represent a single plant over an entire growing season are essential for robust breeding program. At present, neither of WUE measurements is directly suitable for selecting plants from segregating populations. Plants discriminate against the heavy isotope of carbon during photosynthesis. Theory has been well developed that links the variability in WUE with carbon isotope discrimination ($\Delta^{13}C$) [117]. The application of $\Delta^{13}C$ as a surrogate for WUE has been verified in several container and field experiments. This technique is being widely adopted as a surrogate for determining the genetic variability in WUE in several crops such as wheat [118–120], groundnut [105, 121–123], rice [124, 125], cowpea [108, 126, 127], and cotton [100, 111, 113, 128].

As expected by theory, several workers found a negative relationship between $\Delta^{13}C$ and WUE among cotton lines *G. Barbadense* [128], *G. hirsutum*, and an interspecific hybrid [100, 111, 129]. However, a positive correlation of $\Delta^{13}C$ with yield and stomatal conductance at single-leaf level was often noticed [100, 129] (Figure 53.6).

Assessment of genetic variability in WUE using $\Delta^{13}C$ revealed significant variability in many crop species [110, 120, 123, 125]. Similarly, a significant genotypic variability in $\Delta^{13}C$ among 158 upland cotton accessions was observed in our center (Figure 53.7) [64].

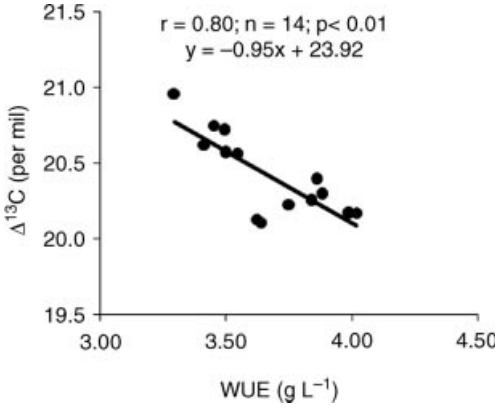

Figure 53.6 Relationship between carbon isotope discrimination ($\Delta^{13}C$) with water use efficiency among 15 cotton lines (*G. hirsutum*) [100].

Besides, the large genetic variability and a low genotype interaction with its environment and a moderate-to-high broad-sense heritability for WUE trait [113, 120, 124, 130, 131] suggest that breeding efforts could be attempted to improve productivity through selection for WUE. For instance, in a wheat breeding program, Richards *et al.* [131] and Condon *et al.* [120, 132] developed varieties with a high WUE to improve productivity under water-limited conditions. However, the yield advantage was shown to decrease when water availability during the cropping season increased [120, 131]. In many species and genotypes, moisture stress induced stomatal closure affecting not only transpiration but also photosynthesis, resulting in significant reduction in total biomass production although there is increase in WUE [61, 133]. Crop lines with such characteristics are often referred to as

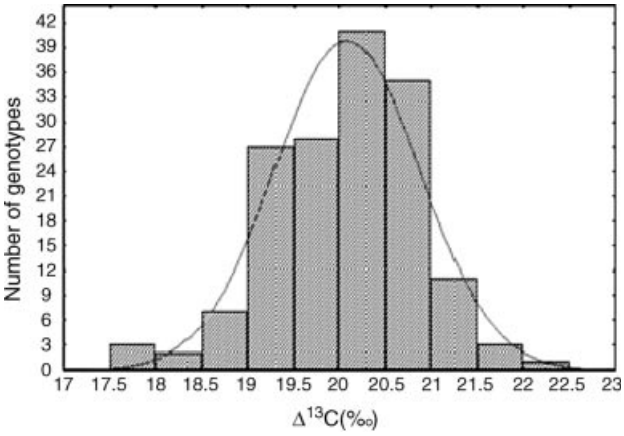

Figure 53.7 Significant genotypic variability observed for $\Delta^{13}C$ among cotton germplasm accessions [64].

Table 53.2 Classification of some crop species as capacity and conductance types.

Capacity (g_m) types		Conductance (g_s) type	
Crop	**Reference**	**Crop**	**Reference**
Groundnut	[104]	Wheat	[141]
Wheat	[119]	Wheat	[142]
Groundnut	[135]	Barley	[143]
Groundnut	[103]	Wheat	[144]
Groundnut	[121]	Wheat	[145]
Groundnut	[136]	Coffee	[146]
Beans	[137]	Cotton	[128]
Grasses	[138]	Cotton	[64]
Canola	[139]	Rice	[124]
Spruce	[140]	Cowpea	[108]

"conductance types" [95, 108, 110]. On the other hand, increase in WUE arises predominantly by maintaining high photosynthetic capacity in a few species and genotypes; selection in such types for higher WUE will not affect the plant biomass and yield that are called "capacity types" [106, 110, 134]. The classification of some crops into capacity and conductance types is provided in Table 53.2.

Cotton is classified as conductance types (Table 53.2), where WUE increases by reducing transpiration through stomatal closure, thus associated with reduction in the biomass produced [132, 147]. Recently, AbouKheir *et al.* [100] demonstrated stable isotopes (^{13}C and ^{18}O) as high-throughput technique for accurate identification of capacity type among cotton genotypes. The carbon and oxygen isotope ratios of plant organic matter integrate the diurnal and seasonal variations in C_i and g_s, respectively [110, 135]. Therefore, demonstration of carbon- and oxygen-stable isotope ratio ($\Delta^{13}C/\Delta^{18}O$) is a good time-averaged estimate of *in vivo* photosynthetic capacity and thus to identify capacity type genotypes.

53.4 Water Conservation Mechanisms

53.4.1 Epicuticular Wax

A variety of functions have been attributed to these waxes related to their involvement in physical and physiological processes, including the reduction of transpiration and gas exchange. Commonly, drought-stressed cotton leaves exhibit an increase in the waxy cuticular layer of the leaf [148, 149] for water conservation. Bondada *et al.* [150] showed that water stress increased total wax concentration in cotton and induced the production of long-chain alkanes whose number and levels were higher in the total wax of the leaf, bract, and boll compared to the corresponding well-watered cotton

plants. It has been reported that cuticular thickness and waxiness of leaf surface are genetically controlled by a large number of genes affecting the transpiration [151].

53.4.2 Cuticular/Nonstomatal Water Loss

Most of the nonstomatal water loss from the leaf would be from the epidermis due to differences in cuticular thickness. It is predominantly influenced by cuticular waxes, which is an intrinsic drought-tolerant character affecting water retention in leaves. The trait has great relevance in water conservation under drought conditions. The genetic variation in cuticular water loss has been reported in crop species and used as selection criterion for drought resistance [152–155]. Genotypic variation in cuticular water loss was assessed among the 158 cotton germplasm accessions in our center by measuring the rate of water loss from excised leaves. Results revealed a considerable genotypic variation among cotton accessions tested in moisture retention capacity at 5 h that ranged from 40.04 to 83.27% (Figure 53.9) [25].

53.5 Cellular-Level Tolerance

53.5.1 Intrinsic Tolerance

Under natural conditions, development of abiotic stress levels is a progressive phenomenon. Accordingly, plants initially experience a milder stress level before being exposed to severe stress. In response to milder stress, several genes have been shown to be either upregulated or downregulated, which leads to the development of a considerable degree of tolerance in plants when stress becomes severe. Such an acclimatization response of plants is termed "acquired tolerance" and it has been demonstrated in several species [156–160] including cotton [161].

The induction of stress triggers several signaling pathways, resulting in the expression of an array of stress-responsive genes and the gene products may alter the physiological and biochemical processes relevant to stress tolerance. While several genes are suppressed, a large number of genes are upregulated in response to stress [162–165]. Drought stress-responsive genes have their own cognate receptors, which operate independently or cooperatively to initiate downstream signaling events. Regulation of both upstream and downstream components such as heat shock protein, protein kinases, secondary messengers (reactive oxygen species (ROS), calcium, etc.), and transcription factors is directly or indirectly involved in establishing the required responses that are well studied [166–168].

On the basis of the concept that seedlings acquire a certain degree of stress tolerance when exposed to milder stress levels, the novel and high-throughput "temperature induction response" (TIR) technique has been developed and validated in many crop species [162, 164, 165] including cotton [161]. The technique involves

Table 53.3 Genetic variability in thermotolerance among 36 *G. hirsutum* genotypes [161].

	Seedling mortality (%)		Reduction in recovery growth (%)	
	Induced	**Noninduced**	**Induced**	**Noninduced**
Mean	49.77 ± 4.2	88.61 ± 2.2	80.54 ± 2.4	96.94 ± 0.6
Minimum	10.00	50.00	43.59	87.22
Maximum	93.30	100.00	96.56	100.00
CD @ 5%	24.63		16.20	

subjecting young seedlings to a mild induction temperature before exposing them to a potentially lethal temperature level. Using the TIR technique, AbouKheir *et al.* [161] observed significant difference in heat stress response among cotton species. Old World cotton (*G. arboreum* and *G. herbaceum*) species showed higher survival percentage and better recovery growth compared to New World cotton (*G. hirsutum and G. barbadense*). Plausibly, it may provide an explanation why diploid cotton types are preferred by farmers in India for cultivation in marginal and drought-prone environments. Many studies have shown that genetic variability is seen only upon stress acclimation treatment prior to severe stress [162, 164, 169–172]. It corroborates the observations of AbouKheir *et al.* [161], who reported a significant genetic variability among 36 upland cotton cultivars screened using TIR technique (Table 53.3), and on the basis of seedling survival and growth after recovery genotypes can be classified into thermotolerant, moderately tolerant, and susceptible (Figure 53.8).

In addition, seedlings exposed to induction treatment before being exposed to severe temperature have higher growth recovery than those seedlings subjected directly to severe temperature stress and also accumulated higher levels of low and high molecular weight HSPs such as HSP18.1, HSP90, and HSP104 [162, 164]. A number of earlier studies have clearly demonstrated that thermotolerance genotypes selected

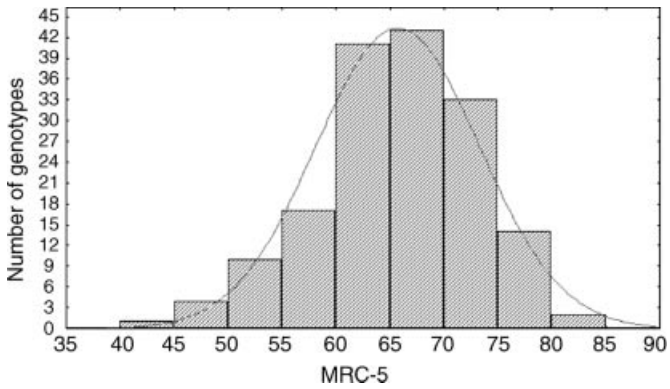

Figure 53.8 Significant genotypic variability in moisture retention capacity at 5 h (MRC-5) among 158 upland cotton accessions [25].

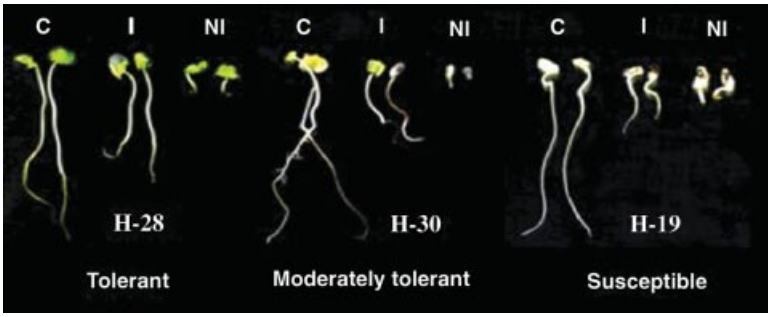

Figure 53.9 Variation in root and shoot growth in tolerant (H-28), moderately tolerant (H-30), and susceptible (H-19) lines after recovery from high-temperature stress.

with TIR technique showed better leaf area, membrane integrity, and thus better recovery growth after exposure to heat stress at whole-plant level. It suggests that the thermotolerance of genotypes analyzed using TIR technique at seedling level may also provide insight into intrinsic tolerance at plant level [162, 164, 165] (Figure 53.9).

53.5.2 Osmotic Adjustment

Osmotic adjustment helps in maintaining turgor and thus tissue metabolism under drought stress conditions [173]. Hare *et al*. [174] reported accumulation of many compatible solutes (i.e., proline, trehalose, and glycine betaine) useful in osmotic adjustment, which also protect plants against water deficit stress by buffering redox reactions by scavenging free radicals, preventing protein degradation, maintaining membrane stability, and mediating signal transduction. Boyer [175] suggested that certain sugars and their derivatives with structures similar to water (e.g., sucrose and trehalose) may hydrogen bond with cell membranes, thus stabilizing the system when water deficits occur. Oosterhuis and Wullschleger [176] found that cotton has more osmotic adjustment capabilities than other major crops and the most important solutes found in cotton leaves are potassium, nitrate, citrate, malate, and sugars, and the last two account for 10–40% of osmotic potential [177].

Eaton and Ergle [178] reported increase in hexose sugars in cotton leaves under moisture-stressed conditions. A decrease in sucrose concentration with concomitant increase in raffinose concentration is known to maximize the secondary wall synthesis in cotton fiber [179]. Cotton has been shown to accumulate an extremely high amount of glycine-betaine compared to other taxa [180]. Trehalose production is induced by a number of stresses including water deficit and salinity in cotton [181]. Trehalose-6-phosphate synthase RNA was found in all tissues tested in both water-stressed and well-watered plants with increased levels of expression in stressed leaves and roots compared to the well-watered controls. Similarly, reduction in osmotic potential was recorded under moisture stress conditions [182]. Singh *et al*. [183] showed an increase in P uptake from a drying soil leads to an increased supply of

osmotically active inorganic solutes for the cells in the growing cotton leaves. As a consequence, plants accumulate both free and bound water, ultimately leading to increased leaf expansion rate. Thus, root traits that enhance the P uptake could be a beneficial mechanism to alleviate drought stress.

Quisenberry *et al.* [184] reported significant differences among cotton cultivars for osmotic potential at zero turgor, further suggesting genotypic variation in osmoregulation in cotton. However, a significant negative correlation between shoot dry weight and increased osmoregulation was reported from the same group, implying that if cotton genotypes are selected on the basis of the degree of osmotic adjustment under water deficit stress alone, a decrease in potential growth could be a possibility in selected species. Evidently, documented reports suggest that relationship between osmotic adjustment and yield were inconsistent, which can be positive [82], negative [184, 185], or no association [186, 187].

53.5.3
Chlorophyll Fluorescence

F_v/F_m is often used as an early indicator of photosynthetic damage caused by abiotic stress in many crops [188–191]. Burke [192] developed a novel bioassay for the identification of drought stress in cotton that utilizes chlorophyll fluorescence to monitor cell viability under high-temperature dark incubation. Differences between well-watered and drought-stressed plants can be established since, under stress, plants will not mobilize carbohydrate reserves overnight and will therefore maintain higher fluorescence values during high-temperature dark incubation, with the opposite being true for well-watered plants. Normal metabolic processes have been shown to be disrupted by drought leading to a reduction in the translocation of photosynthate from leaves to other plant tissues [193]. The okra leaf genotypes did have 14% greater F'_v/F'_m across treatments compared to the normal leaf cottons. Higher photosynthetic rates per unit leaf area have been observed in okra leaf genotypes [194]. Higher chlorophyll fluorescence values in drought-stressed plants were observed in 20 genotype of diverse germplasm evaluated in a 2 year field evaluation under two water regimes [195], indicating the importance of using this bioassay to evaluate and identify the drought tolerance cotton genotypes.

53.6
Conceptual Approach to Cotton Improvement for Abiotic Stress

A variety of approaches have been used to alleviate the problem of drought stress through the enhancement of adaptive mechanisms of plants. It appears to be a formidable task as drought is a complex abiotic stress controlled by many physiological and biochemical characteristics, besides phenotypic characteristics of plants. Plant breeding, either conventional or molecular breeding, is being extensively used to tailor and develop genotypes with genetically superior traits to enable them to adapt to drought-stress environments. There has been commendable progress made

during the last century by plant breeders in developing drought-tolerant lines/varieties/cultivars of some crops through conventional breeding. However, the approach is highly time consuming and labor and cost intensive. Alternatively, marker-assisted selection (MAS) [196] has been realized to be a more efficient approach, which identifies the usefulness of thousands of genomic regions of a crop under stress conditions. Quantitative trait loci (QTL) for drought tolerance have been identified for a variety of traits in different crops. The development of comprehensive molecular linkage maps and marker-assisted selection has opened up options to pyramid desirable traits to achieve crop drought tolerance. To date, such crop improvement programs have been hampered by significant genetic × environment interaction, large number of genes encoding yield and use an inappropriate mapping population involved in mapping of QTL for better growth and yield under water deficit conditions. Advancement of scientific knowledge on genomics and proteomics has been increasingly beneficial for precise molecular breeding and transformation approaches in crop improvement. With the available tools and techniques, the realization of trait pyramiding poses a daunting challenge. Therefore, the emphasis must be placed to devising appropriate strategies and tools for precise trait pyramiding to improve drought tolerance.

The first step in this direction would be to identify genes and QTL that control the specific traits of relevance to drought tolerance and such desirable traits' variability among the genotypes. Hence, precise identification of trait donor parents/lines/genotypes is critical. It forms the basis for the development of genetic and genomic resources for focused molecular breeding and transgenic program. Despite the realized potential of molecular breeding strategy, the success has not been encouraging. It is mainly attributed to the inability to identify robust QTL due to the nonavailability of large number of codominant marker systems such as SSR. Therefore, emphasis has to be on generation of large genomic resources of marker systems (SNP and SSR) followed by devising robust and high-throughout phenotyping techniques.

Transgenics is yet another potential option to introduce novel alleles for improving drought tolerance of crop. In recent years, several transgenic programs have provided leads in improving crop adaptations, even at field level, suggesting the potentials and prospects of trait pyramiding by introducing regulatory genes with abilities to upregulate the several downstream functional genes. Suggestively, choice of regulatory genes and development of multigene cassettes with pathway-engineered genes appear to be an effective drought-tolerant trait pyramiding strategy by transgenic approach.

53.7
Potential of Genomic Resources for the Enhancement of Drought Tolerance

In the last decade, extensive research in genetics and genomics has improved our understanding of structure and function of plant genomes and has integrated basic knowledge to enhance drought stress tolerance through molecular and transgenic approaches. Cotton genetic maps have been developed for various *Gossypium* crosses, mostly between *G. barbadense* species and *G. hirsutum*. These maps collectively

include more than 5000 public DNA markers (~3300 RFLPs, 700 AFLPs, more than 2000 SSRs, and 100 SNPs). Many thousands of additional SSRs have been described [197], but only subsets of these have been mapped [198–200]. The other aspect of genomic resources is transcriptome, a cDNA sequence, which is beginning to receive wide attention. However, available knowledge of the *Gossypium* spp. transcriptome is heavily biased toward genes expressed in fibers. The total number of *Gossypium* ESTs available is 378 184 in different cotton species. On the basis of the current knowledge on cotton genomic resources, Gene Chip (Affymetrix) has been constructed with 23 977 probe sets representing 21 854 cotton transcripts.

Over the past two decades, whole-genome sequencing has been achieved in several organisms including plant species, and it is clear that most of the major crop species will also be sequenced in near future including cotton genome. In cotton, however, there is need to sequence not only cultivated (tetraploid) genotypes but also their diploid progenitors, to understand how tetraploid cottons have come to "transgress" the productivity and quality of their progenitors. As a long-term goal of characterizing the spectrum of diversity among eight genome types, the first genetically anchored whole-genome physical map of a cotton species (*G. raimondii*) was built through integration of agarose-based fingerprinting and high information content fingerprinting [201] since *G. raimondii* (D genome) has the smallest genome (60% of the "A" genome) with 880 Mb, and a detailed genetic linkage map is available. Studies are in progress on the species *G. arboreum* and *G. hirsutum*, the next priorities after *G. raimondii* in the international strategy for characterizing the spectrum of *Gossypium* diversity [202]. The economic importance of cotton fibers and scientific interest in polyploidy suggest an ultimate goal of sequencing a *G. hirsutum* tetraploid. The possibility of intergenomic concerted evolution, much like the presence of recently amplified repetitive DNA families, may tend to support the need for a BAC-based rather than a whole-genome shotgun approach. Using a finished diploid genome as a template and guide, a BAC-based sequence of a tetraploid will elucidate the types and frequencies of changes that have distinguished polyploid from diploid cottons. An effective utilization of the existing genomic resources for precise identification of QTL/genes of relevance for enhancing the adaptive capability to moisture-limited conditions is need of the day.

53.7.1 Molecular Breeding

The marker technology has enabled breeders to utilize DNA markers for selecting desirable plants without exposing them to a critical and growth-limiting environmental conditions [203]. The range of DNA markers that can be easily used is quite extensive. The techniques utilize the polymorphisms in the actual DNA sequence of plant germplasm and identify the variations and use different DNA markers such as RFLP, AFLP, SSR, and so on [204–206]. Such DNA markers have been developed and used in cotton for fingerprinting [207, 208], linkage map construction [198, 199, 209–212], gene mapping [213, 214], and genetic diversity studies [209, 210]. Several studies and reviews have reported that QTL may play a critical role in mitigating abiotic stress in

Table 53.4 Few QTL affecting traits associated with drought tolerance in cotton.

Traits	Marker system	Mapping population	Remarks	References
Fiber length (6)[a], length uniformity (7), elongation (9), strength (21), fineness (25), and color (11)	RFLP markers	214 F_3 families (*G. hirsutum* × *G. barbadense*)	The F_3 family grown in two water regimes well-watered treatment (receiving 300 mm) and the water-limited treatment (receiving about 40–50% of that quantity)	[224]
Lobe length (3), lobe width (2), leaf-lobe angle (3), main sub-lobe number (1)	RFLP markers	180 F_2 (*G. hirsutum* × *G. barbadense*)	QTL affecting leaf-lobe length and width was found at the lower end of chromosome 15 corresponding approximately to the location of the "Okra-leaf" mutation [226]	[225]
Trichome density (4)	RFLP markers	F_2 (*G. hirsutum* × *G. barbadense*)	119–150 from each four F2 population were developed from crossing four *G. hirsutum* with common *G. barbadense*	[226]
Lobe length (10), lobe width (13), lobe angle (6), and leaf chlorophyll content (2)	SSR markers	BC (*G. hirsutum* × *G. barbadense*)	The genetic map was constructed using 590 SSR markers	[227]
Stomatal conductance (2)	RAPD and SSR markers	118 $F_{2.3}$ (*G. hirsutum* × *G. barbadense*)	The genetic analysis was done in F_2 individual and repeated in F_3 families	[228]
Low OP (11), high $\delta^{13}C$ (11), canopy temperature (4), chlorophyll-a (3), and chlorophyll-b (4)	RFLP markers	900 F_2 and 214 F_3 families (*G. hirsutum* × *G. barbadense*)	Two irrigation regimes were used, well watered and water limited	[229, 230]
Root weight (1)	RFLP and SSR markers	The two F_2 populations consisted of 138 and 107 progenies (*G. hirsutum* × *G. barbadense*)	Major QTL for root weight was identified on chromosome 7	[231]

a) Number in parenthesis in the first column indicates number of identified QTL.

many crop species. However, in cotton a majority of QTL identified in genetic mapping were associated with agronomic and fiber quality [212, 213, 215–223]. On the other hand, there are a few studies on QTL affecting traits associated with drought tolerance listed in Table 53.4.

Genomic approaches offer unique opportunities to dissect quantitative traits into their single genetic determinants (QTL), thus enabling transfer of specific genomic regions between different genetic backgrounds through MAS [196]. In cotton, MAS has been used in backcrossing transgenes from transformed cultivars to the elite cultivars and further genetic advancement of cultivars. QTL for yield and drought-related physiological traits such as osmotic potential (OP), carbon isotope ratio ($\delta^{13}C$), and leaf chlorophyll content have been introgressed via MAS to 12 NIL obtained from elite cultivars of the two cotton species *G. barbadense* cv. F-177 and *G. hirsutum* cv. Siv'on. Among nine NILs introgressed with OP QTL, five NILs exhibited significant reduction in OP under water-limited conditions and/or improved OA during two field trials. Similarly, among six NILs introgressed with high $\delta^{13}C$ (as indirectly measurement of WUE) only four showed significantly higher $\delta^{13}C$ under moisture stress conditions. Likewise, only two out of three NILs showed higher chlorophyll content [182].

Over the past decades, conventional linkage analysis (biparenteral QTL mapping) is being successfully used for dissection of trait inheritance mechanism. The approach has facilitated the identification of major genes and QTL in plant and animal species, particularly in model organisms. However, efficient gene discovery with this approach will probably continue to be largely limited to loci that have a large effect on quantitative variation. Although biparenteral mapping is used extensively to identify QTL associated with different traits, it is time consuming and takes several years to develop populations for fine scale mapping. Apart from inherently poor resolution (long-distance associations between marker and QTL, for example, 10 cM) resulting from limited meiotic crossover events in pedigreed populations, developing large full sib families for each major gene/trait may not be practical for plant genetic improvement, particularly in tree crops. A more efficient approach that does not require generation of large pedigreed mapping populations with higher resolution is therefore needed to complement conventional QTL mapping strategy. Recently, a population genomics tool termed "association mapping" [232, 233] has been developed. Association mapping seeks to identify specific functional variants (i.e., loci, alleles, etc.) linked to phenotypic differences in a trait, to facilitate detection of trait causing DNA sequence polymorphisms, and/or selection of genotypes that closely resemble the phenotype. In cotton (*Gossypium* spp.), association studies are very limited [25, 234–236].

Abdurakhmonov *et al.* [234] reported the extent of genome-wide LD in upland cotton (*G. hirsutum*) and association mapping of fiber quality traits using 95 microsatellite markers in a total of 285 exotic *G. hirsutum* accessions. A genome-wide average of LD declined ($r^2 \geq 0.1$) within the genetic distance at $<$10 cM in the landrace stocks of germplasm and $>$30 cM in varietal germplasms. Furthermore, genome-wide LD ($r^2 \geq 0.2$) reduced on an average $\sim$1–2 cM in the landrace stock germplasms and 6–8 cM in varietal germplasms. Recently, association mapping for fiber-quality traits was conducted using 202 microsatellite markers in 335 *G. hirsutum* germplasm grown in two diverse environments. The result indicates that genome-wide average of LD ($r^2 \geq 0.1$) extended up to a genetic distance of 25 cM in assayed cotton variety accessions. Genome-wide LD ($r^2 \geq 0.2$) was reduced to $\sim$5–6 cM

Table 53.5 Summary of the association mapping of root traits, $\Delta^{13}C$, TDM, and other associated physiological traits using MLM and GLM tests in TASSEL.

Traits	No. of significant associations	
	Experimental location 1	Experimental location 2
Plant height @ 100 DAS	27	26
Root length	15	23
Total root volume	28	27
Leaf weight	36	36
Total root weight	34	29
Stem weight	33	23
TDM	29	24
$\Delta^{13}C$	23	20
SCMR	25	39
SLA	19	20
LA	39	33
Root/LA	33	28
TDM/LA	26	30
Root/shoot	32	24

Source: AbouKheir [25]; GLM: general linear model; MLM: mixed linear model; Location 1: Nagpur; Location 2: Bengaluru.

providing evidence for the potentiality of association mapping of agronomically important traits in cotton [235]. Similarly, Kantartzi and Stewart [236] evaluated 56 *G. arboreum* (diploid cotton) germplasm accessions for eight fiber characters that genotyped with 98 SSR markers. A total of 30 marker-trait associations were identified with 19 SSR markers located on 11 chromosomes and identified marker-trait associations ($P = 0.05$) for all traits evaluated.

The first study on LD-based association mapping for root traits and $\Delta^{13}C$ was done in our center, using 152 upland cotton germplasm accessions grown under two diverse agroclimatic locations in India. The results revealed the genome-wide average of LD ($r^2 \geq 0.1$) extended up to a genetic distance of 25–30 cM. Interestingly, it is the first study showing SSR markers associated with root traits and $\Delta^{13}C$ trait in upland cotton (Table 53.5). Although majority of SSR markers had significant associations only in one experimental location, a few of them showed significant association in both the locations with some traits, which can be potential markers for effective MAS program in cotton [25].

53.7.2
Transgenics to Improve Abiotic Tolerance

Plants must adapt to abiotic stress in order to survive; thus, a number of genes are upregulated and/or downregulated as a response to stress conditions. These stress-responsive genes are broadly classified as genes thought to be involved in protecting stressed cells (functional gene) and genes implicated in regulation of signal transduction

Table 53.6 Genes used in transformation to enhance stress tolerance in cotton.

Functional genes	Remarks	References
1. *Antioxidants*		
Superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR)	Enhanced recovery of photosynthesis in plants exposed to chilling temperatures and high light intensity	[239, 240]
Glutathione *S*-transferase (GST)	Seedlings did not show improved tolerance to salinity, chilling conditions, or herbicides	[241]
2. *Membrane transporters*		
Tonoplast Na^+/H^+ antiporters (NHX1)	Transgenic plants had more biomass produced and fibers under salinity stress compared to the wild type	[242]
H^+-PPase gene (TsVP)	Transgenic plants showed high shoot and root growth, better photosynthetic performance, and accumulated more Na^+, K^+, $Ca2^+$, Cl^-, and soluble sugars in their root and leaf tissues under salinity conditions compared to the wild type	[243, 244]
3. *Osmotic potential*		
Choline monooxygenase (CMO)	The osmotic potential, electrolyte leakage, and malondialdehyde (MDA) accumulation were significantly lower in leaves of the transgenic lines than in wild type after salt stress. Net photosynthesis and F_v/F_m was less affected by salinity stress in transgenic	[245]
4. *Protein kinases*		
SOS2	Transgenic plant had better salt tolerance than the wild type	[246]
5. *Transcription factors*		
GF14λ, encodes 14-3-3 group of regulatory proteins	Overexpression of GF14λ in cotton conferred a "stay-green" phenotype under well-watered conditions and displayed increase water stress tolerance and maintained higher photosynthetic rates under conditions of low water availability	[247]
ABF3	Transgenic cotton plants enhanced expression of ABA-responsive genes under noninducting conditions, better root development, and enhanced survival under severe water deficit conditions	[246]

and expression (transcription factor genes) [237, 238]. These genes have been used extensively to enhance the stress tolerance in several of plant species. Some of the relevant genes, which enhance the stress tolerance in cotton, are provided in Table 53.6.

Although the first transgenic cotton plant (Bt cotton) was released and commercially cultivated since 1996, to date there is no transgenic cotton for abiotic stress tolerance. This is perhaps due to the complexity of abiotic stress and lack of efficient transformation technique in cotton. Therefore, there is a need to develop efficient transformation technique that can be used effectively in cotton. Recently, efficient

embryogenic calli-based method of obtaining transgenic cotton has been standardized in two chinese cultivars [248]. In cotton, due to its recalcitrance and poor regeneration, there has been limited progress in achieving effective transformation. A recent alternative method, tissue culture-independent *in planta* transformation strategy, has been successfully used for transformation in cotton [249].

53.8 Summary

Drought is perhaps the most complex abiotic stress and the varied adaptive mechanisms evolved by plants have made the task of improving drought tolerance a formidable challenge. Imperatively, projected prevalence and spread of drought stress due to water deficit situations worldwide has prioritized the efforts in crop improvement program to dissect the molecular and physiological basis of adaptations to drought stress and/or water-limited conditions by various crops. Past research efforts have emphasized on improving constituent drought tolerance traits through a "trait-based breeding" strategy. Thus, the major emphasis has to identify drought-tolerant traits such as root traits, WUE, and so on, and develop precise and high-throughput phenotyping technique for screening a large number of germplasm to select trait donor lines that can be utilized in breeding program. Modern genomic techniques have aided tremendously in identifying stress QTL and diagnostic DNA markers for specific stress-related traits in a number of crops including cotton. Improvement-specific traits by introducing new alleles by transgenic approach would be an essential option to pyramid traits for improving the adaptation. Furthermore, turning the gene-tagging efforts from biparental crosses to germplasm collection and from traditional linkage mapping to linkage disequilibrium (LD)-based association study holds promise for most effective utilization of *ex situ* conserved natural genetic diversity of cotton germplasm resources irrespective of their origin. The application of LD-based association mapping for cotton will not only accelerate the MAS programs in cotton but also add to our knowledge and understanding of the cotton genome and its evolution. Furthermore, genomic resources with drought-tolerant traits can be compatibly used for pyramiding to superior genetic background, which facilitates genetic improvement for higher yield of cotton germplasm and enhanced adaptability to drought stress conditions.

References

1 Fryxell, P.A. (1968) *Bot. Gazette*, **129**, 296–308.

2 Fryxell, P.A. (1979) *The Natural History of the Cotton Tribe*, Texas A&M University Press, College Station, TX.

3 Fryxell, P.A. (1992) *Rheedea*, **2**, 108–165.

4 Wendel, J.F., Brubaker, C., Alvarez, I., Cronn, R., and Stewart, J.M.C.D., (2009) Evolution and Natural History of the Cotton Genus In: Genetics and Genomics of Cotton, A.H. Paterson (ed.), Plant Genetics and Genomics: Crops and

Models 3, Springer ScienceþBusiness Media, LLC (2009), p3–22.
5 Ulloa, M., Stewart, J.M.C.D., Garcia, C.E.A. *et al*. (2006) *Genet. Resour. Crop Evol.*, **53**, 653–668.
6 Brubaker, C.L. and Wendel, J.F. (1994) *Am. J. Bot.*, **81**, 1309–1326.
7 ICAC (2005) *Cotton Production Practices. Technical Information Section*, International Cotton Advisory Committee, Washington DC, USA.
8 ICAC (2004) *Cotton: Review of the World Situation*, International Cotton Advisory Committee, Washington D.C., p. 20.
9 FAO (Food and Agriculture Organization) (2006) FAO Production Year Book, FAO.
10 Khadi, B.M., Santhy, V., and Yadav, M.S. (2010) Cotton: an introduction, in *Cotton, Biotechnology in Agriculture and Forestry 65* (ed. U.B. Zehr), Springer, Berlin, pp. 1–14.
11 FAO (Food and Agriculture Organization) (2007) FAO Production Year Book, FAO.
12 Bot, A.J., Nachtergaele, F.O., and Young., A. (2000) *World Soil Resources Reports 90. Land and Water Development Division*, FAO, Rome.
13 Sharp, R.E., Poroyko, V., Hejlek, L.G. *et al*. (2004) *J. Exp. Bot.*, **55**, 2343–2351.
14 Paterson, A.H., Lander, E.S., Hewitt, J.D. *et al*. (1988) *Nature*, **335**, 721–726.
15 Rosenow, D.T., Quisenberry, J.E., Wendt, C.W. *et al*. (1983) *Agric. Water Manage.*, **7**, 207–222.
16 Hearn, A.B. (1969a) *J. Agric. Sci. Camb.*, **73**, 75–86.
17 Marani, A. and Levi., D. (1973) *Agron. J.*, **65**, 637–641.
18 Constable, G.A. and Hearn, A.B. (1981) *Irrigation Sci.*, **3**, 17–28.
19 Pace, P.F., Cralle, H.T., El-Halawany, S.H.M. *et al.* (1999) *J. Cotton Sci.*, **3**, 183–187.
20 Constable, G.A. and Rawson, H.M. (1980) *Aust. J. Plant Physiol.*, **7**, 89–100.
21 Krieg, D.R. (1981) Leaf development and function as related to water stress, in *Proceedings of the Beltwide Cotton Production Reserach Conference* (ed. J.M. Brown), New Orleans, LA, 4–8 January, 1981, National Cotton Council, Memphis, TN, pp. 41–42.
22 Marani, A., Baker, D.N., Reddy, V.R. *et al*. (1985) *Crop Sci.*, **25**, 798–802.
23 Krieg, D.R. (1997) *Proceedings of the Beltwide Cotton Production Reserach Conference*, National Cotton Council, Memphis, TN, pp. 1337–1347.
24 Cook, C.G. (1985) MSc thesis, Texas A&M University, College Station, TX.
25 AbouKheir, E. (2010) PhD thesis, University of Agricultural Sciences, Bengalaru, India.
26 Hearn, A.B. (1969) *J. Agric. Sci. Camb.*, **73**, 87–97.
27 Grimes, D.W. and Yamada, H. (1982) *Crop Sci.*, **22**, 134–139.
28 Parida, A.K., Dagaonkar, V.S., Phalak, M.S. *et al*. (2007) *Plant Biotech. Rep.*, **1**, 37–48.
29 Parida, A.K., Dagaonkar, V.S., Phalak, M.S. *et al*. (2008) *Acta Physiol. Plant.*, **30**, 619–627.
30 Klepper, B., Taylor, H.M., Huck, M.G. *et al*. (1973) *Agron. J.*, **65**, 307–310.
31 Taylor, H.M. and Klepper, B. (1974) *Agron. J.*, **66**, 584–588.
32 Ball, R.A., Oosterhuis, D.M., and Mauromoustakos, A. (1994) *Agron. J.*, **86**, 788–795.
33 Fernández, C.J., Cothren, J.T., and McInnes., K.J. (1996) *J. Plant Nutr.*, **19**, 595–617.
34 Mauney, J.R. (1986) Vegetative growth and development of fruiting sites, in *Cotton Physiology* (eds J.R. Mauney and J.McD. Stewart), The cotton Foundation, Memphis, TN, pp. 11–28.
35 Jackson, B.S. and Gerik, T.J. (1990) *Agron. J.*, **82**, 483–488.
36 Morrow, M.R. and Krieg, D.R. (1990) *Agron. J.*, **82**, 52–56.
37 Baker, D.N., Bruce, R.R., and McKinion., J.M. (1973) Proceedings of the 1972 Cotton Production Research Conference, pp. 110–114.
38 Wullschleger, S.D. and Oosterhuis, D.M. (1990) *Crop. Sci.*, **30**, 1259–1264.
39 Gerick, T.J., Faver, K.L., Thaxton, P.M. *et al*. (1996) *Crop. Sci.*, **36**, 914–921.
40 McMichael, B.L., Jordan, W.R., and Powell, R.D. (1973) *Agron. J.*, **65**, 202–204.
41 Hearn, A.B. (1976) *J. Agric. Sci. Camb.*, **86**, 257–269.

42 Guinn, G. and Mauney, J.R. (1984a) *Agron. J.*, **76**, 90–94.

43 Guinn, G. and Mauney, J.R. (1984b) *Agron. J.*, **76**, 94–98.

44 Antony, A.K. and Kutty, K.E. (1975) *Ind. J. Agr. Sci.*, **45**, 199–203.

45 Longenecker, D.E. and Erie, L.J. (1968) Irrigation water management, in *Advances in Production and Utilization of Quality Cotton: Principle and Practices* (eds F.C. Elliot, M. Hoover, and W.K. Poter), Iowa State University Press, San Diego, CA, pp. 321–345.

46 Bilbro, J.D. (1962) *Texas Agri. Exp. Station M.P.*, **611**, 1–8.

47 Levi, A., Ovnat, L., Paterson, A.H. *et al.* (2009a) *Plant Sci.*, **177**, 88–96.

48 Mahmood, S., Irfan, M., Raheel, F. *et al.* (2006) *Int. J. Agri. Biol.*, **8** (6), 796–800.

49 Cutler, J.M. and Rains, D.W. (1977) *Crop Sci.*, **17**, 329–335.

50 Radin, J.W. and Mauney, J.R. (1986) The nitrogen stress syndrome, in *Cotton Physiology*, The Cotton Foundation, Memphis, pp. 91–105.

51 McMichael, B.L. and Hesketh, J.D. (1977) Proceedings of the 31st Cotton Physiology Conference, pp. 62–63.

52 Hearn, A.B. (1979) *Outlook Agric.*, **10**, 159–166.

53 Cothren, J.T. (1999) Physiology of the cotton plant, in *Cotton* (eds C.W. Smith and J.T. Colthern), John Wiley & Sons, Inc., New York, pp. 207–268.

54 Krieg, D.R. (1983) *Agric. Water Manage.*, **7**, 249–263.

55 Krieg, D.R. (1986) Feedback control and stress effects on photosynthesis, in *Cotton Physiology* (eds J.R. Mauney and J.McD. Stewart), The Cotton Foundation, Memphis, TN, pp. 227–242.

56 Jones, H.G. (1973) *New Phytol.*, **72**, 1095–1105.

57 Iqbal, J., Reddy, O.U.K., EL-Zik, K.M. *et al.* (2001) *Theor. Appl. Genet.*, **103**, 547–554.

58 Rungis, D., Llewellyn, D., Dennis, E.S. *et al.* (2005) *J. Agric. Res.*, **56**, 301–307.

59 Miflin, B. (2000) *J. Exp. Bot.*, **51** (342), 1–8.

60 Collins, N.C., Tardieu, F., and Tuberosa, R. (2008) *Plant Physiol.*, **147**, 469–486.

61 Blum, A. (2009) *Field Crops Res.*, **112**, 119–123.

62 Passioura, J.B. (1976) *Aust. J. Plant Physiol.*, **3**, 559–565.

63 Passioura, J.B. (1986) *Aust. J. Plant Physiol.*, **13**, 191–201.

64 AbouKheir, E., Sheshshayee, M.S., Udayakumar, M. *et al.* (2008) AAB International Conference on Resource Capture by Crops: Integrated Approach, 14–16 September 2008, University of Nottingham at Sutton Bonington Campus, UK.

65 Andries, J.A., Jones, J.E., Sloane, L.W. *et al.* (1969) *Crop Sci.*, **9**, 705–710.

66 Heitholt, J.J. (1993) *Crop. Sci.*, **33**, 486–490.

67 Kerby, T.A., Buxton, D.R., and Matsuda, K. (1980) *Crop Sci.*, **20**, 208–213.

68 Pettigrew, W.T., Heitholt, J.J., and Meredith, W.R. (1993) *Agron. J.*, **85**, 821–825.

69 Kerby, T.A. and Buxton, D.R. (1978) *Agron. J.*, **70**, 535–538.

70 Wells, R. and Meredith, W.R. (1986) *Crop Sci.*, **26**, 223–228.

71 Meredith, W.R. (1984) *Crop Sci.*, **24**, 855–857.

72 Stiller, W.N., Reid, P.E., and Constable, G.A. (2004) *Agron. J.*, **96**, 656–664.

73 McMichael, B.L. (1986) Growth of roots, in *Cotton Physiology* (eds J.R. Mauney and J.McD. Stewart), The Cotton Foundation, Memphis, TN, pp. 29–38.

74 McMichael, B.L., Burke, J.J., Berlin, J.D. *et al.* (1985) *Environ. Exp. Bot.*, **25**, 23–30.

75 McMichael, B.L., Oosterhuis, D.M., Zak, J.C. *et al.* (2010) Growth and development of root systems, in *Cotton Physiology* (eds J.McD. Stewart, D.M. Oosterhuis, J.J. Heitholt, and J.R. Mouney), Springer Science, pp. 57–71.

76 Hons, F.M. and McMichael, B.L. (1986) *Field Crops Res.*, **13**, 147–158.

77 McMichael, B.L. and Quisenberry, J.E. (1991) *Environ. Exp. Bot.*, **31**, 461–470.

78 Ludlow, M.M. and Muchow, R.C. (1990) *Adv. Agron.*, **43**, 107–153.

79 Prior, S.A., Rogers, H.H., Runion, G.B. *et al.* (1995) *J. Environ. Qual.*, **24**, 678–683.

80 Plaut, Z., Carmi, A., and Grava, A. (1996) *Irrigation Sci.*, **16**, 107–113.

81 Huang, B., Duncan, R.R., and Carrow., R.N. (1997) *Crop Sci.*, **37**, 1863–1869.

82 Blum, A. (2005) *Aust. J. Agri. Res.*, **56**, 1159–1168.

83 Martinez, F., Merino, O., Garcia, M.D. *et al.* (1998) *Plant Soil*, **201**, 209–216.

84 Tuberosa, R., Sanguineti, M.C., Landi, P. *et al.* (2002) *Plant Mol. Biol.*, **48** (5/6), 697–712.

85 Drouet, J.L., Pagès, L., and Serra, V. (2005) *Euro. J. Agron.*, **22**, 185–193.

86 Udayakumar, M., Rao, R.C.N., Wright, G.C. *et al.* (1998) *J. Plant Biol.*, **1**, 69–75.

87 Taylor, H.M., Upchurch, D.R., Brown, J.M. *et al.* (1991) Some methods of root investigation, in *Plant Roots and Their Environment* (eds B.L. McMichael and H. Persson), Elsevier Science Publishers, Inc., New York.

88 Sheshshayee, M.S., AbouKheir, E., Sreevathsa, R. *et al.* (2011) Sustaining crop productivity through improvement in root traits: phenotyping and breeding for relevant traits, in *Root Genomics* (ed. A.D. de Oliveira), Springer, The Netherlands, pp. 205–232.

89 Basal, H., Bebeli, P., Smith, C.W. *et al.* (2003) *Crop Sci.*, **43**, 1983–1988.

90 Roark, B. and Quisenberry, J.E. (1977) Proceedings of the Beltwide Cotton Production Research Conference, Atlanta, GA. 10–12 January, 1977, National Cotton Council of America, Memphis, TN, pp. 49–50.

91 Quisenberry, J.E., Jordan, W.R., Roark, B.A. *et al.* (1981) *Crop Sci.*, **21**, 889–895.

92 Craig, L. and Gordon, L.I. (1965) Deuterium and oxygen-18 variations in the ocean and the marine atmosphere, in *Proceedings of a Conference on Stable Isotopes in Oceanographic Studies and Paleotemperatures* (ed. E. Tongiorgi), Spoleto, Italy, pp. 9–130.

93 Flanagan, L.B., Bain, J.F., and Ehleringer, J.R. (1991) *Oecologia*, **88**, 394–400.

94 Flanagan, L.B., Phillips, S.L., Ehleringer, J.R. *et al.* (1994) *Aust. J. Plant Physiol.*, **21**, 221–234.

95 Farquhar, G.D. and Lloyd, J. (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere, in *Stable Isotopes and Plant Carbon–Water Relations* (eds J.R. Ehleringer, A.E. Hall, and G.D. Farquhar), Academic Press, San Diego, pp. 47–70.

96 Farquhar, G.D., Cernusak, L.A., and Barnes, B. (2007) *Plant Physiol.*, **143**, 11–18.

97 Gonfiantini, R., Gratziu, S., and Tongiorgi, E., Technical Report Series No. 206. Isotopic Atomic Energy Commission, Vienna, 405–410. (1965)

98 DeNiro, M.J. and Epstein, S. (1979) *Science*, **204**, 51–53.

99 Sheshshayee, M.S., Bindumadhava, H., Ramesh, R. *et al.* (2005) *J. Exp. Bot.*, **56**, 3033–3039.

100 AbouKheir, E., Prasad, T.G., Sheshshayee, M.S. *et al.* (2010) *Asian Aust. J. Plant Sci. Biotech.*, **4** (1), 12–18.

101 Sinclair, T.R., Tanner, C.B., and Bennett, J.M. (1984) Water-use efficiency in crop production. *BioSci.*, **34**, 36–40.

102 Tanner, C.B. and Sinclair, T.R. (1983) Efficient water use in crop production: research or re-search? in *Limitations to Efficient Water Use in Crop Production* (eds H.M. Taylor, W.R. Jordan, and T.R. Sinclair), The American Society of Agronomy (ASA), Crop Science Society of America (CSSA), and Soil Science Society of America (SSSA) Madison, Wisconsin, USA, pp. 1–25.

103 Hubick, K.T., Farquhar, G.D., and Shorter, R. (1986) *Aust. J. Plant Physiol.*, **13**, 803–816.

104 Wright, G.C., Hubick, K.T., and Farquhar, G.D. (1988) *Aust. J. Plant Physiol.*, **15**, 815–825.

105 Wright, G.C., Rao, R.C.N., and Farquhar, G.D. (1994) *Crop. Sci.*, **34**, 92–97.

106 Udayakumar, M., Sheshshayee, M.S., Nataraj, K.N. *et al.* (1998) *Current Sci.*, **74**, 994–1000.

107 Rao, R.C.N., Williams, J.H., Wadia, K.D.R. *et al.* (1993) *Ann. Appl. Biol.*, **122**, 357–367.

108 Ashok, R.S., Aftab Hussein, I.S., Wright, G.C. *et al.* (1999) *Funct. Plant Bio.*, **26**, 503–510.

109 Ebdon, J.S., Petrovic, A.M., and Dawson, T.E. (1998) *Crop Sci.*, **38** (1), 157–162.

110 Sheshshayee, M.S., Bindumadhava, H., Shankar, A.G. *et al.* (2003) *J. Plant Biol.*, **30** (2), 253–268.

111 Saranga, Y., Flash, I., and Yakir, D. (1998) *Crop Sci.*, **38**, 782–787.

112 Pettigrew, W.T. (2004b) *Crop Sci.*, **44**, 1265–1272.

113 Stiller, W.N., Read, J.J., Constable, G.A., and Reid, P.E. (2005) *Crop. Sci.*, **45**, 1107–1113.

114 Baker, D.A. (1984) Water relations, in *Advanced Plant Physiology* (ed. M.B. Wilkins), Pitman, London.

115 Voloudakis, A.E., Kosmas, S.A., Tsakas, S. *et al.* (2002) *Funct. Plant Biol.*, **29**, 1237–1245.

116 Basal, H., Smith, C.W., Thaxton, P.S. *et al.* (2005) *Crop Sci.*, **45**, 766–771.

117 Farquhar, G.D., Ehleringer, J.R., and Hubick, K.T. (1989) *Ann. Rev. Plant Physiol. Plant Mol. Bio.*, **40**, 503–537.

118 Farquhar, G.D. and Richards, R.A. (1984) *Aust. J. Plant Physiol.*, **11**, 539–552.

119 Condon, A.G., Farquhar, G.D., and Richards, R.A. (1990) *Aust. J. Plant Physiol.*, **17**, 9–22.

120 Condon, A.G., Richards, R.A., Rebetzke, G.J. *et al.* (2004) *J. Exp. Bot.*, **55**, 2447–2460.

121 Wright, G.C., Hubick, K.T., Farquhar, G.D. *et al.* (1993) Genetic and environmental variation in transpiration efficiency and its correlation with carbon isotope discrimination and specific leaf area in peanut, in *Stable Isotope and Plant Carbon–Water Relations* (eds J.R. Ehleringer, A.E. Hall, and G.D. Farquhar), Academic Press, San Diego, USA, pp. 247–267.

122 Nageswara Rao, R.C., Udayakumar, M., Farquhar, G.D. *et al.* (1995) *Aust. J. Plant Physiol.*, **22**, 545–551.

123 Bindu Madhava, H., Sheshshayee, M.S., Shashidhar, G. *et al.* (2005) *Curr. Sci.*, **189** (7), 1256–1258.

124 Impa, S.M., Nadaradjan, S., Boominathan, P. *et al.* (2005) *Crop. Sci.*, **45**, 2517–2522.

125 Nadaradjan, S., Impa, S.M., Sheshshayee, M.S. *et al.* (2005) Second International Congress of Plant Physiology, 8–12 January 2003, New Delhi, India.

126 Ismail, A.M. and Hall, A.E. (1992) *Crop Sci.*, **32**, 7–12.

127 Ismail, A.M. and Hall, A.E. (1993) *Crop Sci.*, **33**, 498–503.

128 Lu, C., Richards, G.P., Sharti, M.R. *et al.* (1996) *Aust. J. Plant. Physiol.*, **23**, 127–132.

129 Leidi, E.O., Lopez, M., Gorham, J. *et al.* (1999) *Field Crops Res.*, **61**, 109–123.

130 Rebetzke, G.J., Condon, A.G., Richards, R.A. *et al.* (2002) *Crop Sci.*, **42**, 739–745.

131 Richards, R.A., Rebetzke, G.J., Condon, A.G. *et al.* (2002) *Crop Sci.*, **42**, 111–121.

132 Condon, A.G., Richards, R.A., Rebetzke, G.J. *et al.* (2002) *Crop Sci.*, **42**, 122–131.

133 White, J.W., Castillo, J.A., and Ehleringer, J. (1990) *Aust. J. Plant Physiol.*, **17**, 189–198.

134 Scheindegger, Y., Saurer, M., Bahn, M. *et al.* (2000) *Oecologia*, **125**, 350–357.

135 Hubick, K.T., Shorter, R., and Farquhar, G.D. (1988) *Aust. J. Plant Physiol.*, **15**, 799–813.

136 Rao, R.C.N., Udayakumar, M., Farquhar, G.D. *et al.* (1995) *Aust. J. Plant Physiol.*, **22**, 545–551.

137 White, J.W. (1993) Implications of carbon isotope discrimination studies for breeding common bean under water deficits, in *Stable Isotopes and Plant Carbon–Water Relations* (eds J.R. Ehleringer, A.E. Hall, and G.D. Farquhar), Academic Press, pp. 389–396.

138 Johnson, D.A., Asay, K.H., and Read, J.J. (1993) *Stable Isotopes and Plant Carbon–Water Relations* (eds J.R. Ehleringer, A.E. Hall, and G.D. Farquhar), Academic Press, New York, pp. 269–280.

139 Matus, A., Slinkard, A., and Kessel, C.V. (1995) *Crop Sci.*, **35**, 1267–1271.

140 Sun, Z.J., Livingston, N.J., Guy, R.D. *et al.* (1996) *Plant Cell Environ.*, **19**, 887–894.

141 Condon, A.G., Richards, R.A., and Farquhar, G.D. (1993) *Aust. J. Agri. Res.*, **44**, 1693–1711.

142 Farquhar, G.D., Hubick, K.T., Condon, A.G. *et al.* (1988) Carbon isotope fractionation and plant water use efficiency, in *Stable Isotopes in Ecological Research* (eds P.W. Rundel, J.R. Ehleringer, and K.A. Nagy), Springer, New York, pp. 21–40.

143 Acevedo, E. (1993) Stable isotopes and plant carbon–water relations, in *Stable Isotopes and Plant Carbon–Water Relations* (eds J.R. Ehleringer, A.E. Hall, and G.D. Farquhar), Academic Press, San Diego, USA, pp. 398–417.

144 Ehdaie, B., Hall, A.E., Farquhar, G.D. *et al.* (1991) *Crop Sci.*, **31**, 1282–1288.

145 Richards, R.A. and Condon, A.G. (1993) *Stable Isotopes and Plant Carbon–Water Relations* (eds J.R. Ehleringer, A.E. Hall, and G.D. Farquhar), Academic Press, New York, pp. 451–462.

146 Meinzer, F.C., Rundel, P.W., and Schultz, L.M. (1990) *Cov. Funct. Ecol.*, **4**, 579–584.

147 Richards, R.A. (1996) *Plant Growth Regul.*, **20**, 157–166.

148 Weete, J.D., Leek, G.L., Peterson, C.M. *et al.* (1978) *Plant Physiol.*, **62**, 675–677.

149 Oosterhuis, D.M., Hampton, R.E., and Wullschleger, S.D. (1991) *J. Prod. Agric.*, **4**, 260–265.

150 Bondada, B.R., Oosterhuis, D.M., Murphy, J.B. *et al.* (1996) *Envi. Exp. Bot.*, **36** (1), 61–69.

151 Haque, M.M., Mackill, D.J., and Ingram, K.T. (1992) *Crop Sci.*, **32**, 865–868.

152 Clarke, J.M, and Townley-Smith, T.F. (1986) *Crop Sci.*, **26**, 289–292.

153 Clarke, J.M. (1987) Use of physiological and morphological traits in breeding programmes to improve drought resistance of cereals, in *Drought Tolerance in Winter Cereals* (eds J.P. Srivastiva, E. Proceddu, E. Acevedo, and S. Verma), Wiley Interscience, New York, pp. 171–189.

154 Clarke, J.M., DePauw, R.M., and Townley-Smith, T.F. (1992) *Crop Sci.*, **32**, 723–728.

155 Ahmad, R.T., Malik, T.A., Khan, I.A. *et al.* (2009) *Int. J. Agric. Biol.*, **11**, 235–240.

156 Burke, J.J., O'Mahony, P.J., and Oleiver., M.J. (2000) *Plant Physiol.*, **123**, 575–587.

157 Sun, W., Bernard, C., van de Cotte, B.M. *et al.* (2001) *Plant J.*, **27**, 407–415.

158 Sung, D.Y., Kaplan, F., Lee, K.J. *et al.* (2003) *Trends Plant Sci.*, **8**, 179–187.

159 Larkindale, J., Hall, J.D., Knight, M.R. *et al.* (2005) *Plant Physiol.*, **138**, 882–897.

160 SenthilKumar, M., Kumar, G., Srikanthbabu, V. *et al.* (2006) *J. Plant Physiol.*, **164**, 111–125.

161 AbouKheir, E., Sheshshayee, M.S., Prasad, T.G. *et al.* (2010) *J. Cotton Sci.* (in press).

162 Kumar, G., Krishnaprasad, B.T., Savitha, M. *et al.* (1999) *Theor. Appl. Genet.*, **99**, 359–367.

163 Gurley, W.B. (2000) *Plant Cell*, **12**, 457–460.

164 Srikanthbabu, V., Kumar, G., Krishnaprasad, B.T. *et al.* (2002) *J. Plant Physiol.*, **159**, 535–545.

165 SenthilKumar, M., Srikanthbabu, V., Mohan Raju, B. *et al.* (2003) *J. Exp. Bot.*, **54** (392), 2569–2578.

166 Kuznetsov, V.V., Rakitin, V.Y., and Zholkevich, V.N. (1999) *Physiol. Plant.*, **107**, 399–406.

167 Seki, M., Narusaka, H., Abe, H. *et al.* (2001) *Plant Cell*, **13**, 61–72.

168 Zhu, J.-K. (2002) *Ann. Rev. Plant Bio.*, **53**, 247–273.

169 Krishnan, M., Nguyen, H.T., and Burke, J.J. (1989) *Plant Physiol.*, **90**, 140–145.

170 Uma, S., Prasad, T.G., and Udayakumar, M. (1995) *Ann. Bot.*, **76**, 43–49.

171 Jayaprakash, T.L., Ramamohan, G., Krishna Prasad, B.T. *et al.* (1998) *Ann. Bot.*, **82**, 513–522.

172 Burke, J.J. (2001) *Physiol. Plant.*, **112**, 167–170.

173 Zimmerman, U. (1978) *Ann. Rev. Plant Physiol.*, **29**, 122–148.

174 Hare, P.D., Cress, W.A., and van Staden, J. (1998) *Plant Cell Environ.*, **21**, 535–553.

175 Boyer, J.S. (1996) *Adv. Agron.*, **56**, 187–218.

176 Oosterhuis, D.M. and Wullschleger, S.D. (1988) *Arkansas Farm Res.*, **37**, 12.

177 Cutler, J.M. and Rains., S.W. (1978) *Physiol. Plant.*, **42**, 261–268.

178 Eaton, F.M. and Ergle., D.R. (1948) *Plant. Physiol.*, **23**, 169–187.

179 Murray, A.K. (1999) Myo-inositol, sucrosyl oligosaccharide metabolism and drought stress in developing cotton fibers, *in vivo, in vitro* and in plant, in *Proceedings of the Beltwide Cotton Production Research Conference, January 1999* (ed. J.M. Brown), National Cotton Council, Memphis, TM, pp. 518–520.

180 Gorham, J. (1996) *Phytochemistry*, **43**, 367–369.

181 Kosmas, C., Marathianou, M., Gerontidis, St. *et al.* (2001) *Agric. Water Manage.*, **48**, 61–78.

182 Levi, A., Paterson, A.H., Barak, V. *et al.* (2009b) *Mol. Breeding*, **23**, 179–195.

183 Singh, V., Pallaghy, C.K., and Singh, D. (2006) *Field Crops Res.*, **96**, 199–206.

184 Quisenberry, J.E., Roark, B., and McMichael, B.L. (1982) *Crop Sci.*, **22**, 918–922.

185 Singh, D.P., Chaudhary, B.D., Singh, P., Sharma, H.C., and Karwasra, S.P.S.

(1990) *Drought Tolerance in Oilseed Brassicas and Chickpea*, Haryana Agriculture University, Hisar, India, pp. 1–60.
186 Leport, L., Turner, N.C., French, R.J. *et al.* (1999) *Eur. J. Agron.*, **11**, 279–291.
187 Turner, N.C., Abbo, S., Berger, J.D. *et al.* (2007) *J. Exp. Bot.*, **58**, 187–194.
188 Kuk, Y.I., Shin, J.S., Burgos, N.R. *et al.* (2003) *Crop Sci.*, **43**, 2109–2117.
189 Netondo, G.W., Onyango, J.C., and Beck., E. (2004) *Crop Sci.*, **44**, 806–811.
190 Belkhodja, R., Morales, F., Abadía, A. *et al.* (1999) *Photosynthetica*, **36**, 375–387.
191 Jimenez, M.S., Gonzalez-Rodriguez, A.M., Morales, D. *et al.* (1997) *Photosynthetica*, **33**, 291–301.
192 Burke, J.J. (2007) *Plant Physiol.*, **143**, 108–121.
193 Wilson, R.F., Burke, J.J., and Quisenberry, J.E. (1987) *Plant Physiol.*, **84**, 251–254.
194 Pettigrew, W.T. (2004a) *Photosynthetica*, **42**, 567–571.
195 Longenberger, P.S.,PhD thesis, submitted to the Texas A&M University, USA. (2008)
196 Tuberosa, R. and Salvi, S. (2006) *Trends Plant Sci.*, **11**, 405–412.
197 Xiao, J., Wu, K., Fang, D.D. *et al.* (2009) *J. Cotton Sci.*, **13**, 75–157.
198 Rong, J.K., Abbey, C., Bowers, J.E. *et al.* (2004) *Genetics*, **166**, 389–417.
199 Lacape, J.M., Nguyen, T.B., Thibivilliers, S. *et al.* (2003) *Genome*, **46**, 612–626.
200 Yu, J.W., Yu, S.X., Lu, C.R. *et al.* (2007) *J. Integr. Plant Biol.*, **49**, 716–724.
201 Lin, L., Pierce, G.J., Bowers, J.E. *et al.* (2010) *BMC Genomics*, **11**, 395–411.
202 Chen, Z.J., Scheffler, B.E., and Dennis, E. (2007) *Plant Physiol.*, **145**, 1303–1310.
203 Helentjaris, T., Slocum, M., Wright, S. *et al.* (1986) *Theor. Appl. Genet.*, **72**, 761–769.
204 Vos, P., Hogers, R., Blecker, M. *et al.* (1995) *Nucl. Acids Res.*, **23**, 4407–4414.
205 Ulloa, M., Saha, S., Jenkins, J.N. *et al.* (2005) *J. Hered.*, **96**, 132–144.
206 Paterson, A., Estill, J., Rong, J. *et al.* (2002) *Cotton Sci.*, **14**, 31.
207 Rahman, M., Hussain, D., and Zafar, Y. (2002) *Crop Sci.*, **42**, 2137–2144.
208 Rahman, M., Yasmin, T., Tabassum, N. *et al.* (2008) *Genet. Res. Crop Evo.*, **55**, 331–339.
209 Reinisch, A.J., Dong, J.M., Brubaker, C.L. *et al.* (1994) *Genetics*, **138**, 829–847.
210 Ulloa, M., Meredith, W.R., Shappley, Z.W. *et al.* (2002) *Theor. Appl. Genet.*, **104**, 200–208.
211 Zhang, J., Guo, W., and Zhang, T. (2002) *Theor. Appl. Genet.*, **105** (8), 1166–1174.
212 Mei, M., Syed, N.H., Gao, W. *et al.* (2004) *Theor. Appl. Genet.*, **108**, 280–291.
213 Shappley, Z.W., Jenkins, J.N., Zhu, J. *et al.* (1998) *J. Cotton Sci.*, **4**, 153–163.
214 Ulloa, M. and Meredith, W.R. (2000) *J. Cotton Sci.*, **4**, 161–170.
215 Jiang, C.X., Wright, R.J., El-Zik, K.M. *et al.* (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 4419–4424.
216 Kohel, R.J., Yu, J., Park, Y.H. *et al.* (2001) *Euphytica*, **121**, 163–172.
217 Zhang, T.Z., Yuan, Y.L., Yu, J. *et al.* (2003) *Theor. Appl. Genet.*, **106**, 262–268.
218 Lin, Z., He, D., Zhang, X. *et al.* (2005) *Plant Breed.*, **124**, 180–187.
219 Zhang, L., Cao, W., Zhang, S. *et al.* (2005) *Acta Phytoecol. Sci.*, **29**, 266–273 (in Chinese with an English abstract).
220 Shen, X., Guo, W., Zhu, X. *et al.* (2005) *Mol. Breed.*, **15**, 169–181.
221 Shen, X., Guo, W., Lu, Q. *et al.* (2007) *Euphytica*, **155**, 371–380.
222 Park, Y.H., Alabady, M.S., Ulloa, M. *et al.* (2005) *Mol. Genet. Genomics*, **274**, 428–441.
223 Lacape, J.M., Nguyen, T.B., Courtois, B. *et al.* (2005) *Crop. Sci.*, **45**, 123–140.
224 Paterson, A.H., Saranga, Y., Menz, M. *et al.* (2003) *Theor. Appl. Genet.*, **106**, 384–396.
225 Jiang, C., DelMonte, T.A., Paterson, A.H. *et al.* (2000) *Theor. Appl. Genet.*, **100**, 409–418.
226 Wright, R.J., Thaxton, P.M., El-Zik, K.H., and Paterson, A.H. (1999) *J. Hered.*, **90**, 215–219.
227 Song, X.L., Wang, K., Guo, W.Z. *et al.* (2005) *Acta Bot. Sin.*, **47** (11), 1382–1390.
228 Ulloa, M., Zeiger, E., Lu, Z. *et al.* (2000) *J. Cotton Sci.*, **4**, 10–18.
229 Saranga, Y., Menz, M., Jiang, C.X. *et al.* (2001) *Genome Res.*, **11**, 1988–1995.
230 Saranga, Y., Jiang, C.X., Wright, R.J. *et al.* (2004) *Plant Cell Environ.*, **27**, 263–277.
231 Shen, X., Van Becelacre, G., Kumar, P. *et al.* (2006) *Theor. Appl. Genet.*, **13** (8), 1539–1549.

232 Chakraborty, R. and Weiss, K.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9119–9123.
233 Kruglyak, L. (1999) *Nat. Gene.*, **22**, 139–144.
234 Abdurakhmonov, I.Y., Kohel, R.J., Yu, J.Z. *et al.* (2008) *Genomics*, **92**, 478–487.
235 Abdurakhmonov, I.Y., Saha, S., Jenkins, J.N. *et al.* (2009) *Genetica*, **136**, 401–417.
236 Kantartzi, S.K. and Stewart, J.M. (2008) *Plant Breed.*, **127**, 173–179.
237 Shinozaki, K. and Yamazuchi-Shinozaki, K. (1996) *Curr. Opin. Biotech.*, **7**, 161–167.
238 Bartels, D. and Sunkar, R. (2005) *Crit. Rev. Plant Sci.*, **24**, 23–58.
239 Payton, P., Allen, R.D., Trolinder, N. *et al.* (1997) *Photosyn. Res.*, **52**, 233–244.
240 Payton, P., Webb, R.P., Kornyeyev, D. *et al.* (2001) *J. Exp. Bot.*, **52**, 2345–2354.
241 Light, G.G., Mahan, J.R., Roxas, V.P. *et al.* (2005) *Planta*, **222**, 346–354.
242 He, C., Yan, J., Shen, G. *et al.* (2005) *Plant Cell Physiol.*, **46**, 1848–1854.
243 Lv, S., Zhang, K., Gao, Q. *et al.* (2008) *Plant Cell Physiol.*, **49** (8), 1150–1164.
244 Lv, S.L., Lian, L.J., Tao, P.L. *et al.* (2009) *Planta*, **229**, 899–910.
245 Zhang, H., Dong, H., Li, W. *et al.* (2009) *Mol. Breed.*, **23**, 289–298.
246 Allen, R.D. (2010) Opportunities for engineering abiotic stress tolerance in cotton plants, in *Cotton, Biotechnology in Agriculture and Forestry 65* (ed. U.B. Zehr), Springer, Berlin, pp. 127–160.
247 Yan, J.Q., He, C.X., Wang, J. *et al.* (2004) *Plant Cell Physiol.*, **45**, 1007–1014.
248 Zhang, W.X., Nie, Y., and Luo, X. (2005) *Plant Breed.*, **124**, 142–146.
249 Keshamma, E., Rohini, S., Rao, K.S. *et al.* (2008) *J. Cotton Sci.*, **12**, 264–272.

54
Tea: Present Status and Strategies to Improve Abiotic Stress Tolerance

Sanjay Kumar, Asosii Paul, Amita Bhattacharya, Ram Kumar Sharma, and Paramvir Singh Ahuja

Tea (*Camellia sinensis* (L.) O. Kuntze) is sensitive to several abiotic factors including low temperature, drought, frost, hail, and waterlogging. The published work largely relates to low temperature and drought, which are the most prominent environmental cues impacting tea survival and productivity. Low temperature leads to winter dormancy through a complex network wherein genes related to cell rescue, defense, and chaperones were upregulated, and the genes associated with cell cycle and DNA processing were downregulated. A positive correlation with the oxidative stress and winter dormancy necessitated modulating oxidative stress for regulating winter dormancy. Chaperonic activities and oxidative stress have also been reported to play prominent roles in modulating drought response of tea. Importantly, drought stress impaired catechins accumulation by downregulating several genes of the pathway. It is a critical parameter with the background that catechins are one of the important components determining the quality of black tea. In the area of genome mapping, bulk segregant analysis followed by complete genotyping of pseudo-test progeny of two heterozygous parental clones for yield, drought, and other important traits identified 260 informative RAPD and AFLP markers. Transgenic tea has been developed by *Agrobacterium* and biolistic gun methods. Tea overexpressing osmotin is a success story that opened up a new path of developing tea plants with improved traits of choice. Developments in systems biology, next-generation sequencing, microRNAs, epigenetics, transcriptomics, metabolomics, proteomics, molecular markers, and transgenic technologies offer immense opportunities for understanding tea response to varied abiotic stresses followed by utilizing the outcome for tea improvement.

54.1
Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is one of the most important perennial crops of commerce grown in a wide range of climatic conditions from Georgia (formerly a part of the USSR) in the north (45°N) to South Africa (30°S) and from New Guinea (150°E)

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in the east to Argentina (60°W) in the west [1, 2]. Recent statistics (2009) suggested that tea occupied 2.99 million hectare to yield 3.88 million ton [3]. Mid-term projections generated by FAO World Tea Model indicate that India would continue to be the largest producer of black tea with an output of 1.2 million ton by 2017, followed by Kenya and Sri Lanka with projected production of 344 000 ton and 341 000 ton, respectively. China is expected to follow with a projected production of 312 000 ton. [3].

Tea, as a commercial crop, includes several species within the genus *Camellia* in the family Theaceae. Three different types of tea, namely, China (*C. sinensis*), Assam (*C. assamica*), and the Cambod (*C. assamica* subsp. *Lasiocalyx*) contribute significantly to the entire genetic pool of cultivated tea worldwide [4]. Although, tea is grown primarily for beverage, recent reports showing its therapeutic value makes it an ideal health drink.

The apical buds and the associated two leaves (TAB), which are used for commercial tea production, contain many compounds of medicinal importance, such as volatile oils, vitamins, minerals, purines, alkaloids (e.g., caffeine), and polyphenols (catechins and flavonoids) [5]. Tea has been shown to have anticancerous [6], antioxidant [7], antimicrobial [8], and anticataract [9] properties. Epidemiological surveys have associated tea drinking with reduced risk of cardiovascular diseases, while studies in cell culture and animal models indicated a potentially beneficial effect of tea on gene transcription, cell proliferation, and other molecular functions. In the past few years, clinical studies revealed several physiological responses to tea that might be relevant to the promotion of health and the prevention or treatment of some chronic diseases [10].

54.2
Major Abiotic Stresses Affecting Tea Yield and Quality

Tea is an evergreen, perennial plant that grows naturally as tall as 15 m. However, under cultivated condition, the bush height is maintained at 60–100 cm to ease harvesting. Tea of commerce is produced from TAB; thus, vegetative growth of TAB is of prime importance for optimized yield. Environment coupled to genetic potential is critical to realize the yield potential. Unfavorable environmental conditions will impact growth processes, thereby the yield. The effects could be on canopy development, and production and partitioning of nutrients and the dry matter [11]. While nutrient application can be controlled, there may not be control on temperature, water availability, and solar radiations and hence these parameters result in abiotic stress in tea, as in any other crop/plant species. Tea industries across the world are always trying to increase production potential by optimizing management practices and improving cultivars.

Tea yield is sensitive to a number of abiotic factors, namely, low temperature, drought, frost, hail, soil pH, waterlogging, and light intensity. However, most of the published work relates to winter dormancy and drought stress, which are probably the major conspicuous factors impacting tea productivity and survival.

54.2.1 Winter Dormancy

Dormancy is defined as "the inability to initiate growth from meristems or other organs and cells with the capacity to resume growth, under favorable conditions" [12]. Dormancy is well-studied in seeds, tubers, corms, and apical buds of several plant species. However, the literature is scanty in tea, in spite of the fact that dormancy, particularly the winter dormancy, is a prominent and obvious phenomenon. It is one of the major bottlenecks to tea growing regions that lie away from the equator. Winter dormancy is characterized by the diminished growth of TAB during winter months. The growth rate of TAB during the dormant season can be reduced to 20% of the mean growth rate recorded, during the active growth period; during the peak of the growing season, TAB growth rate reached 900% of the growth rate during the dormant season [13]. Tea cultivated close to the equator produces almost the same yield every month, but farther from the equator, winter harvest gradually declines and at latitudes beyond about 16°, there is almost complete winter dormancy [1]. The crop remains dormant for a period up to 6 months in countries such as Turkey and Iran, whereas dormancy in Argentina and Mauritius may span over a period of 2–4 months. In Northeast India tea bushes remain dormant during winter for 2–3 months, whereas in Himachal Pradesh (Northwest India), dormant period spans for 5–6 months. Winter dormancy thus affects yield, land use, infrastructure, and manpower.

54.2.1.1 Physiological and Biochemical Basis

Data showed a positive correlation between free abscisic acid (ABA) and winter dormancy in tea [14]; the levels of free gibberellins, on the contrary, remained low during winter dormancy [15]. It was suggested that an altered balance of the endogenous growth regulators could lead to winter dormancy in tea. The efforts to modulate winter dormancy in tea by altering the interactions of plant growth regulators, however, yielded inconsistent results [1], possibly because of a lack of understanding of the mechanism underlying winter dormancy in tea.

Low temperatures prevailing during the period of winter dormancy, either alone or in combination with high solar radiation, can induce oxidative stress in tea. Oxidative stress has been proposed to affect growth and development of plants by limiting biomass production and hence productivity [16–18].

Results on 11 clones of tea, representing China and Assam types, showed that as the atmospheric temperature declined, net photosynthetic rates (P_N) declined (up to 286%) along with reduction in F_v/F_m ratios [19]. Reactive oxygen species (ROS), estimated as superoxide radicals, also increased in all the tea clones during the period of low temperature. The decrease in the rate of photosynthesis and F_v/F_m ratio (a measure of chlorophyll fluorescence) in all clones with concurrent increase in the ROS suggested imposition of oxidative stress in tea during winter months. A strong correlation was obtained between the levels of free radicals, P_N, F_v/F_m and the rate of bud growth. These results suggested that tea does experience oxidative stress during

winter months. Studies across the selected 11 clones showed distinct differences observed for this stress.

Clones with shorter dormancy periods exhibited higher induction of antioxidative enzymes and vice-versa. Results suggested that efficient scavenging of ROS was a desirable feature in tea because it leads to lower ROS accumulations during winter months and was associated with reduced period of winter dormancy [19]. It was also shown that the clones with lower period of winter dormancy exhibited lesser cellular damage in response to low temperature [20]. Glutathione reductase and Mn-superoxide dismutase [21] were identified to be critical in modulating the process of winter dormancy in tea. One of the remarkable features of the identified Mn-superoxide dismutase was its optimal temperature of functionality, which was at 0°C.

54.2.1.2 **Molecular Approaches**

Cultivated species of tea has a diploid chromosome numbers of 30 [22] carrying genome size of 4.0 GB [23]. Compared to 125 Mb for *Arabidopsis thaliana* [24] and 382.17 Mb for rice [25], genome of tea can be considered as a large genome. Transcriptome analysis offers a convenient route for such a large genome and this can be achieved by a number of methods such as using genome analyzers and analysis of expressed sequence tags (EST). As on January 2011, NCBI databank had a total of 13 054 ESTs chiefly contributed by Anhui Agricultural University, Hefei, P.R. China; Tea Research Association, Assam, India; Tea Research Institute, Chinese Academy of Agricultural, Zhejiang, P.R. China; Faculty of Horticulture, Uttar Banga Krishi Viswavidyalaya, West Bengal, India; Institute of Himalayan Bioresource Technology, Palampur, H.P, India; Tea Research Association Tocklai/North Bengal Agricultural, Assam, India; National Institute of Agricultural Biotechnology, Kyeonggi, Korea; and College of Horticulture, Northwest A&F University, Shaanxi, P.R. China. There are 14 more institutes/groups that have contributed ESTs to the database; however, their contribution was less than 1% of the total EST submissions and hence not detailed in this chapter.

First genomic work on molecular aspects in tea was published by Park *et al*. [26] to describe 588 clones of a subtracted library of tea. Thereafter, Chen *et al*. [27] reported random sequencing of a cDNA library from tea describing 1684 high-quality ESTs. The first winter dormancy-related EST data set at NCBI database was submitted between March 2008–Jan 2009 by the Institute of Himalayan Bioresource Technology, Palampur (accession numbers FF682697–FF682833; GH454303–GH454326; FE942774–FE943102). These ESTs were created by analyzing a subtracted library of tea prepared using RNA of the actively growing (AG) and the winter-dormant (WD) tissue. A prominent difference between the two libraries (Figure 54.1, unpublished work; analyzed on the basis of EST analysis) was the presence of comparatively large "cell rescue, defense and virulence" class (47%) in WD tissue comprising sequences encoding late embryogenesis abundant protein family, dehydrin (*Deh*), and metallothionein-like protein; a larger proportion of "cell rescue and defense" class was also reported in the dormant cambial meristems of *Populus tremula* [28] and the dormant buds of *Quercus petraea* (Matt.) Liebl. [29] and *Rubus idaeus* L. [30]. "Cell cycle and DNA processing" class was the major category (22%) in the AG tissue as evidenced by the large number of sequences for histone proteins. The number of genes involved in

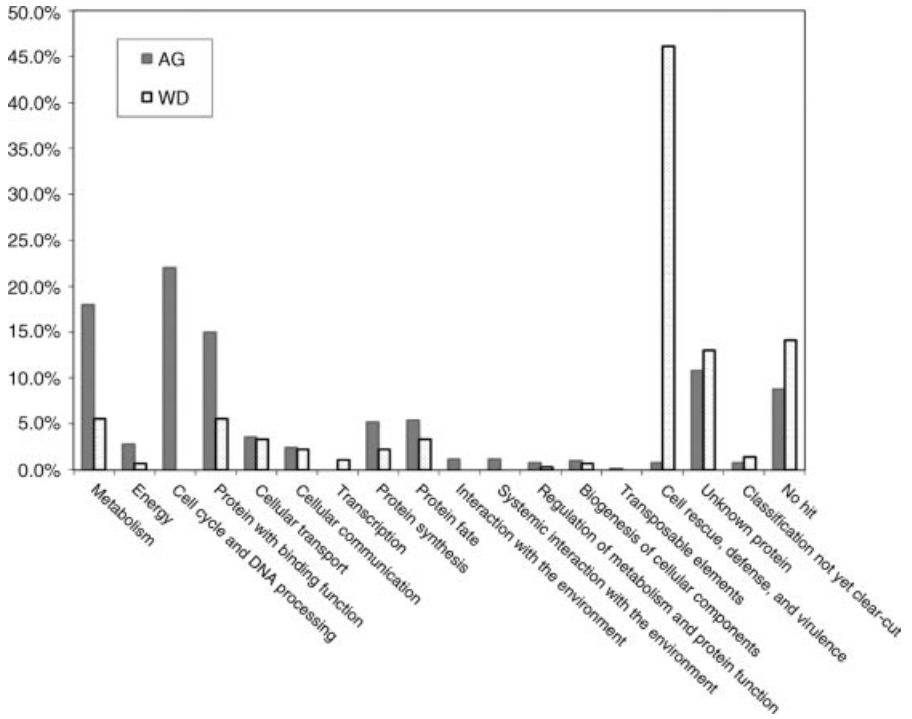

Figure 54.1 Functional classification of clones obtained in subtracted libraries. WD and AG represent the genes upregulated during winter dormancy and the period of active growth, respectively. The clones were grouped into functional classes based on the MIPS classification system [28].

metabolic activity (18%) was six times higher in AG compared to that observed for WD (5.6%). This suggested the need to produce larger amounts of metabolites for the newly forming and dividing cells of the actively growing meristems during PAG. Another dominated class of genes in AG was the "protein with binding function" dominated by *chlorophyll a/b binding protein (CAB)* (54 out of 75 sequences), which accounted for 10.8% of the total sequences in AG. Since CAB is involved in photosynthetic processes, data suggested reduction in net photosynthesis rate and photochemical efficiency of photosystem (PSII) during WD in tea as shown previously as well [19]. The remaining functional classes were comparable in size (in terms of number of transcripts) in AG and WD, though the type of transcript that constituted a particular functional class was different in the two transcriptomes. The combined AG and WD sequences in other functional categories were as follows: energy, cellular transport, cellular communication, transcription, protein synthesis, protein fate, interaction with the environment, regulation of metabolism and protein function, biogenesis of cellular components, transposable elements, and unknown protein.

Analysis of subtracted library also showed that the sequence complexity of the subtracted library representing the genes expressed during WD was less compared to

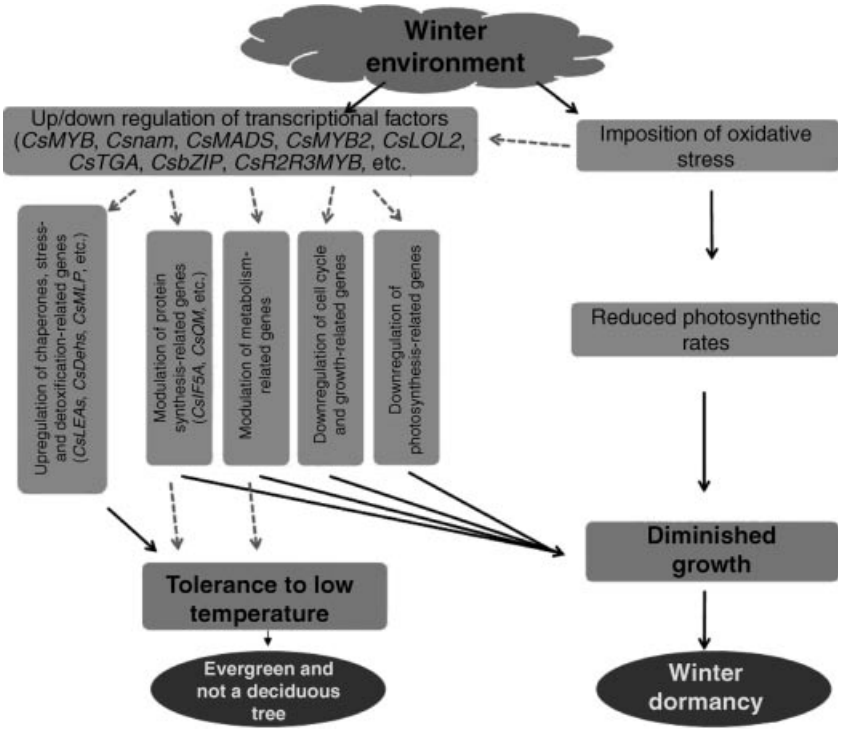

Figure 54.2 Possible mechanism of winter dormancy and evergreen nature of tea tree (19–21; winter dormancy-related ESTs available at NCBI). Solid lines with arrows suggest direct interaction, whereas dashed lines with arrows suggest possible interactions.

the subtracted library representing the genes expressed in AG tissue. Reduced transcriptome complexity during WD might be due to reduction in the number of genes involved in the processes typical for actively growing meristems such as cell division, expansion, and differentiation. For example, a significant reduction in the complexity of the transcriptome in *Populus tremula* L. was observed during cambial dormancy [28]. Thus, the available knowledge on winter dormancy in tea can be summarized as Figure 54.2, which suggests that winter environment-mediated imposition of oxidative stress coupled with reduction in cell division and associated activities might be responsible for winter dormancy in tea. The evergreen nature to this tree species could be attributed to the expression of a large number of genes associated with imparting stress tolerance. These processes might be mediated through several transcription factors.

54.2.2 Drought Stress

Tea is a perennial tree, and as such, encounters a large number of environmental stresses throughout its life span [31]. Drought effect on tea has been reported in

numerous tea growing areas of the world with yield penalty ranging from 14 to 33% [1, 2, 32–36]. Drought also leads to about 6–19% plant deaths depending upon the clone and the severity of the drought [37]. Generally, tea tolerates drought, but at the expense of photosynthates. If the moisture stress prolongs, defoliation and death of plants occurs [35]. Proline accumulation has been shown as one of the mechanisms of drought tolerance in tea; generally, the drought-tolerant clones had significantly higher proline content compared to susceptible ones [38–40]. Though indirect, the data suggest that proline accumulation is related to nonenzymatic detoxification of free radicals [41].

It is expected that with climate change, the main tea growing areas will experience an increase in the length of dry seasons per year and warmer temperatures or extreme rainfall intensity [42]. Thus, drought damages in tea producing regions are expected to increase in the years to come that would lead to greater economic, social, and environmental problems. Tolerance to drought stress is a key factor governing performance of tea in different environments.

Some genetic improvement for water-limited environments has been achieved in tea through breeding and improved crop husbandry. At least part of the slow progress in improving the genetic foundation of dehydration tolerance in tea has been due to a lack of sufficient genetic information about genes that govern this complex trait and its component secondary traits [43]. Research has shown that drought tolerance varies considerably between genotypes of tea [31, 32, 44–47], providing a good basis for investigating the genetic architecture and adaptive responses to water stresses.

Institutes/groups working on tea have realized drought as a major problem limiting productivity and hence the efforts have been to screen the available germplasm for tolerance to drought and to develop germplasm through conventional breeding approaches. For example, Tea Research Association, Tocklai [48], listed 164 clones of tea describing various attributes including response to drought for majority of the clones. TV1, TV17, TV19, TV20, TV23, Garboo parbat 19, Thowra2/11, Bormajan19, and Dhul41 were designated as drought-tolerant clones. Similarly, UPASI Tea Research Foundation [49] recommended clones for drought-prone areas, which include UPASI-2, UPASI-9, ATK-1, TRI-2025, UPASI-20, UPASI-26, UPASI BSS, and BSS-2.

The rapid advances in genomics technologies have led to an increased understanding of global gene expression in plants. As water is lost from the cell, regulatory processes are initiated that adjust cellular metabolism to the new cellular conditions [50]. At the same time, growth inhibition and alterations of developmental pathways will result in changes in gene expression. It is estimated that approximately 12% of the total plant transcriptomes are differentially expressed during water stress [51]. Many of these water deficit-induced genes encode gene products predicted to protect cellular function. Genes that function during changes in metabolism, regulation, signaling, and recognition of stress are also expected to be induced, but fewer of these classes of genes have been identified in tea.

By January 2011, the number of tea ESTs in the National Center of Biotechnology Information (NCBI) database was 13 054 among which 2387 were related to drought. Molecular study on the response of tea to drought was initiated by Sharma and

Kumar [45] reporting three drought-modulated ESTs through differential display of mRNA that represented a PR-5 family, calsequestrin, and novel genes. *PR-5* gene exhibited strong upregulation under drought stress compared to the irrigated control, and in plants during recovery from the drought, suggesting its involvement in the process of drought. Drought also accompanied decrease in nitrogen assimilation in tea as evidenced through downregulation of glutamine synthetase [52].

At least in embryo of tea seeds, proteomic work was carried out to understand the desiccation-responsive proteome [53]. Desiccation-mediated accumulation of hydrogen peroxide was noticed with concomitant increase in the activities of ascorbate peroxidase and superoxide dismutase. Upregulated proteins were associated with defense response, metabolism, and redox status. Data suggested that higher accumulation of ROS that is not efficiently scavenged by increased levels of antioxidant enzymes, impacted seed viability. And hence reduction of ROS was proposed as an efficient way to reduce desiccation damage and improve germination rates. Thus, management of oxidative stress appeared a common strategy either for winter dormancy or for improvement of desiccation stress in tea [19–21].

54.2.2.1 Drought Stress and Catechin Metabolism

Catechins are polyphenols that determine the quality of made tea and particularly for manufacture of black tea, higher catechin content is a desirable feature. Apart from the impact of drought on productivity, there is also an interest to study the impact of drought and the associated cues on catechin metabolism. Drought stress has been shown to impair catechin accumulation in tea (Figure 54.3) and similar was the response to exogenous application of hormone ABA [54–57]. Detailed analysis on various genes of the pathway included work on *phenylalanine ammonia-lyase* (*CsPAL*), *cinnamate 4- hydroxylase* (*CsC4H*), *p-coumarate:CoA ligase* (*Cs4CL*), *flavanone 3-hydroxylase* (*CsF3H*), *dihydroflavonol 4-reductase* (*CsDFR*), and *anthocyanidin reductase* (*CsANR*) [54–58]. *Cs4CL*, *CsPAL*, *CsC4H*, *CsF3H*, *CsDFR*, and *CsANR* exhibited downregulation in response to drought stress and ABA. Gene expression correlated positively with catechin content suggesting that the impact of drought on catechins was at the level of transcript. Lower catechin production under drought stress could be an adaptive feature with the background that higher catechin level (200 μM and above) severely retarded growth and development of plants [59].

54.2.2.2 Molecular Markers for Improvement of Drought Stress

The processes of domestication and selection have resulted in a drastic narrowing of genetic base of majority of crop species [60]. Breeding of such cultivars has led to greater susceptibility of many crops to biotic and abiotic stresses [61]. Furthermore, the genetic bottlenecks arising due to transitions between wild genotypes and landraces to early domestications and then modern cultivars have led to a significant loss of potentially useful genes. The negative impact of utilizing varieties with a narrow genetic base was demonstrated by the fact that of all released clonal teas for commercial use in Kenya, 67% share the same female parent clone TRFK 6/8, which has been observed to be susceptible to root knot nematodes [62]. Furthermore, abiotic and biotic stresses and narrow genetic background may result in huge losses in tea

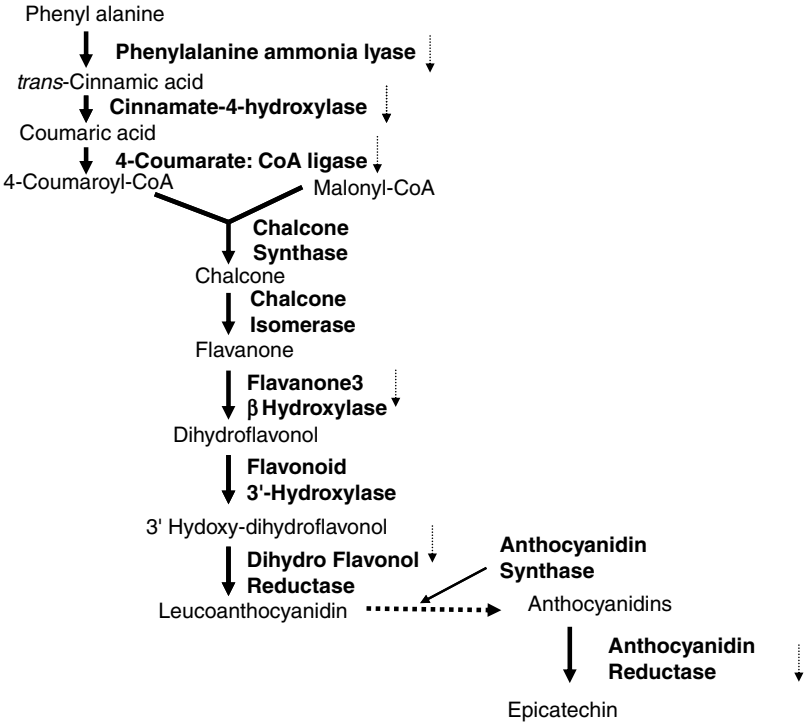

Figure 54.3 Catechin biosynthesis pathway in tea adapted from previous work (55–58). The gene downregulated under drought stress/ABA are marked with ↓.

production. Therefore, there is a thrust for molecular marker-based rationalization of breeding strategy to identify stress-tolerant (biotic and abiotic) and distantly related parents to serve as candidate genotypes for intraspecific and/or interspecific hybridization in the existing gene banks [63]. This strategy will broaden the genetic base as well as introgression of new genes and alleles controlling useful traits that were otherwise missing in the base populations.

Bandhopadhyay [64] discussed current status of various molecular markers that could be employed for genetic improvement of tea.

Most comprehensive molecular fingerprinting studies of 1644 Indian tea accessions in a national network project coordinated by IHBT suggest that the Indian tea gemplasm at national level is highly heterozygous [65]. However, amplified fragment length polymorphism fingerprinting study by Sharma *et al.* [66] revealed considerable genetic diversity (GS) among tea accessions associated with trait attributes [SMP, TRI-2043, UPASI-19, SA-6, and BS-26 (average genetic distance (AGS), 0.56; resistant to blister blight); UPASI-26 and UPASI-27 (AGS, 0.62; resistant to frost); UPASI-1, UPASI-10, UPASI-26, and CH-1 (AGS, 0.53; tolerant to wind); TV-9 (tolerant to waterlogging); and 26 accessions with an AGS of 0.57 (tolerant to drought)]. Overall, 44% genetic diversity was recorded in these accessions. Since these accessions were highly heterogeneous and were clustered in different groups

and subgroups, can be considered as potential parental groups for combing of these traits in quality tea accessions through controlled crossing in future tea improvement programs. These inferences based on AFLP markers, however, can be validated with set of highly polymorphic microsatellite or simple sequence repeat (SSR) markers in near future, as there are about 324 SSR markers that have been reported in tea [67–73]. Realizing the genome complexity, coupled with the reported genetic linkage map length [43, 74], a large repository of microsatellite markers might be required not only to saturate existing genetic maps but also for future quantitative trait loci (QTL) mapping and marker-assisted selection in tea.

Combining abilities and genetic parameters of parental groups for yield, drought tolerance, and quality-related traits in *C. sinensis* suggested significant phenotypic variation in the targeted traits measured among the progeny and their parents [75, 76]. Genetic improvement process would have been much enhanced with the availability of genome wide sequence based microsatellite and single nucleotide polymorphism (SNPs) markers. Quantitative trait mapping, however, will be more efficient if molecular markers were assigned to the linkage groups. Such linkage maps for tea were constructed with random amplified polymorphic DNA and AFLP markers and covered 1349.7 cM, with an average distance of 11.7 cM [74]. QTL controlling yield, drought tolerance, and quality traits (percent total polyphenols), fermentability, theaflavins, thearubigins, and pubescence were studied for initiating marker-assisted selection and breeding in tea [77]. Recently, QTL controlling yield, drought tolerance (DT), and quality traits (percent total polyphenols (%TP), fermentability (FERM), theaflavins (TF), thearubigins (TR), and pubescence (PUB) were studied for initiating marker-assisted selection and breeding in tea [77]. Bulk segregant analysis [78] followed by complete genotyping of a pseudo-test progeny of two heterozygous parental clones for yield, drought, and other important traits identified 260 RAPD and AFLP informative markers. Of these 100 markers that showed 1 : 1 segregation, a linkage map was generated with 30 (19 maternal and 11 paternal) linkage groups that spanned 1411.5 cM with mean interval of 14.1 cM between loci. On the basis of the map, QTL analysis was done on data over two sites. A total of 64 putative QTL controlling various traits across the two sites were detected. Of these, QTL linked to YLD-T, YLD-K, DT-K, and PUB were localized at 2 cM, 2.7 cM, 3 cM, and 1.4 cM from markers OPG-07-2800, E-AGC/M-CAG-725, OPT-18-2500, and OPO-02-650, respectively [77].

54.2.3
Transgenic Approaches for Improvement of Abiotic Stress Tolerance in Tea

Since conventional breeding of tea is highly time consuming and labor intensive, crop improvement by transgenic technology has gained considerable popularity over the recent years [79]. Genetic transformation of tea by *Agrobacterium* and biolistic gun methods were attempted by different workers from all across the world [80–88] and the success ranged from production of kanamycin-resistant callus tissue with strong *gus* expression [89] to transgenic plants with *gus* reporter gene and their transfer to polyhouse [90–92]. Mainly, the availability of well-standardized regeneration systems [93–95] made transgenic tea production by these two methods possible.

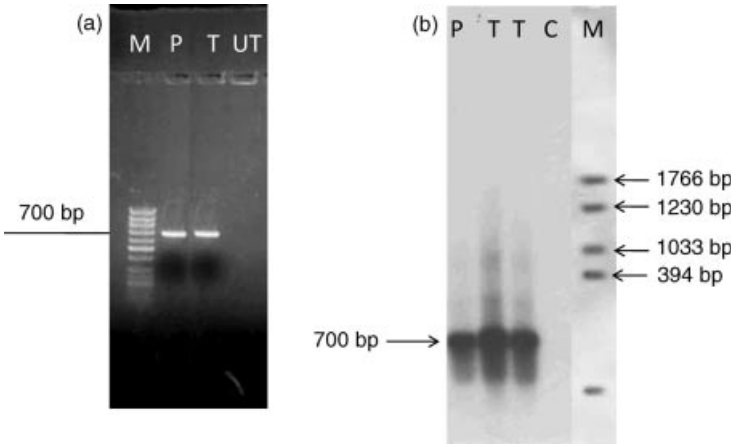

Figure 54.4 (a) Polymerase chain reaction (PCR) amplification of a 700 bp internal fragment of the *osmotin* gene in tea somatic embryos. P, plasmid; T, putatively transformed; UT, untransformed; M, molecular markers [99]. (b) Southern hybridization of *osmotin*-transformed somatic embryos of tea, showing the expected hybridization signals when DIG-labeled osmotin fragments from *Xba* I and *BamH* I sites were used as probes. C, untransformed; T, putatively transformed; P, plasmid harboring *osmotin*; M, molecular markers [99], reprinted with kind permission from Cambridge University Press © 2011.

Methods to overcome the resistance of hosts to *Agrobacterium* infection were also devised and successfully used [96, 97].

These successes opened up an alternative and attractive way of producing tea plants tolerant to abiotic stresses. In this regard, Saini [98] used the biolistic gun method to produce stress-tolerant transgenic plants expressing the *osmotin* gene from *Nicotiana tabacum* cv. White burley. Method optimization included a target distance of 9 cm with burst pressure of 1100 psi to bombard somatic embryos at globular stage of development. These were multiplied in recurrent cycles to produce more than 100 lines of osmotin transformants, which tested positive in both PCR and Southern hybridization (Figure 54.4a and b). When the growth performance of these lines was evaluated, healthier growth and higher multiplication rates were observed compared to either the untransformed control or the ones harboring the *gus* reporter gene. Moreover, when these lines were assessed for their tolerance to abiotic stresses, increased tolerance to desiccation was observed.

Tolerance to desiccation stress is crucial for any developing embryo, particularly in their late maturation stage [100, 101]. This tolerance helps the embryos to remain viable as the mature seeds begin to lose their inherent moisture content and dries up [102, 103]. However, tea seeds are of "recalcitrant type" and have embryos that are highly desiccation sensitive and lose viability on being dried below a critical average of 28% moisture [104]. This is much higher than the level to which most orthodox seeds can be desiccated, that is, 3–5%. Similar to their zygotic counterparts, the somatic embryos of tea are also desiccation sensitive and lack the ability to accumulate

adequate storage reserves during early maturation stage. They also show high frequency of precocious and abnormal germination [105] and are incapable of producing and accumulating sufficient amounts of osmolytes required for cellular adjustments [106]. One way of adapting to abiotic stress is through osmolyte accumulation for membrane stability and osmotic adjustments [107–109]. Thus, tea somatic embryos can serve as ideal system for understanding and devising strategies for overcoming the developmental blocks in recalcitrant type seeds.

In view of this, somatic embryos were used to study the influence of stable integration and expression of *osmotin* gene in the heart or early maturation stage of embryo development. When standard biochemical methods were used, interestingly enough, the somatic embryos showed several fold increase in storage reserves (Figure 54.5a). In contrast, the untransformed somatic embryos without the introduced *osmotin* gene showed poor accumulation of storage reserves. In histochemical studies also, *osmotin*-transformed heart-stage somatic embryos showed abundant depositions of starch, proteins, and also oils in the form of numerous droplets. On the other hand, the untransformed control and the somatic embryos with *gus* and *npt*II genes showed negligible or significantly lower depositions of starch, proteins, and oil globules (Figure 54.5b).

The effect of desiccation on the *osmotin*-transformed somatic embryos was also assessed by first desiccating the somatic embryos in open Petri dishes in laminar hood cabinet for 60 min and then germinating them on basal MS medium supplemented with 1 g/l L-glutamine, 3% sucrose, 0.2 mg/l BA, and 0.1 mg/l IBA as per the method of Bhattacharya *et al.* [110]. A distinct increase in osmotin transcript

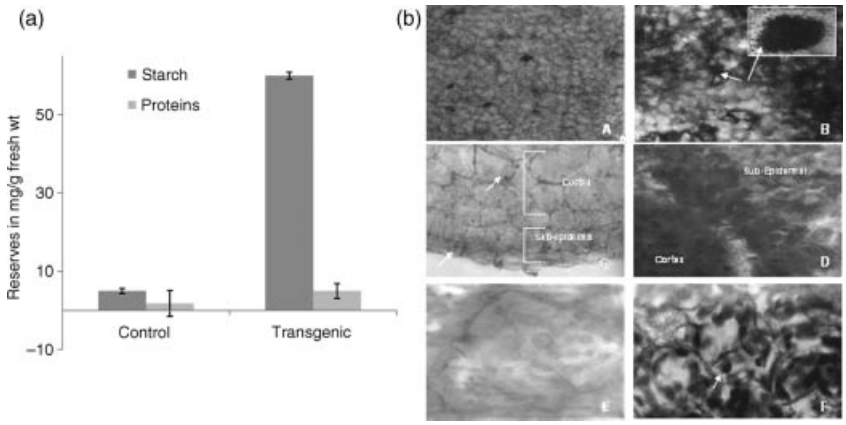

Figure 54.5 (a) Accumulation of reserves in untransformed control and *osmotin*-transformed somatic embryos during heart or early maturation stage of development. (b) Histochemical sections of somatic embryos at heart or maturation stage showing accumulation of storage reserves (A, C, and E) control and (B, D, and F) somatic embryos transformed with *osmotin* gene [99], reprinted with kind permission from Cambridge University Press © 2011).

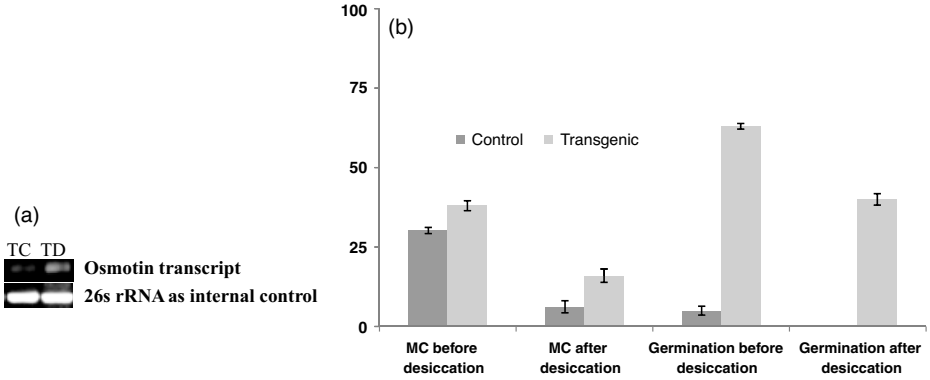

Figure 54.6 Effect of desiccation on the (a) accumulation of *osmotin* transcripts and (b) moisture content and germination of somatic embryos transformed with *osmotin* gene as compared to untransformed control [99], reprinted with kind permission from Cambridge University Press © 2011.

accumulation was observed in the desiccated osmotin transformants compared to the nondesiccated ones (Figure 54.6a). The consequent germination of the *osmotin* transformants was then compared with that of desiccated untransformed control, and also *gus* transformants. The results revealed 40% normal germination in the *osmotin* transformants after 60 min of desiccation, whereas the untransformed control somatic embryos failed to germinate after desiccation and died (Figure 54.6b). However, the germination of the undesiccated *osmotin* transformed somatic embryos was higher, that is, 57–63%, and that of untransformed control and *gus* transformants ranged between 1–5%. The critical moisture content (MC) of the *osmotin*-transformed somatic embryos as influenced by desiccation was also assessed (Figure 54.6b). While the lowering of MC below 20% resulted in total loss of germination capacity in the untransformed control and *gus* transformants, reduction in germination was recorded only in the *osmotin*-transformed somatic embryos. Certain degree of desiccation tolerance was indeed conferred by the introduced *osmotin* gene. This is not surprising because the cationic protein, osmotin, is known to bring about cellular adjustments through osmolyte accumulation and subsequent increase in tolerance to different stresses including the desiccation [111, 112]. These findings revealed that introduction of gene(s) that confer tolerance to abiotic stresses into somatic embryos of plants with recalcitrant seed types can be an important step toward engineering desiccation tolerance, reserve accumulation, and normal germination.

The plants generated from these osmotin-transformed somatic embryos were healthy and grew well under contained polyhouse conditions (Figure 54.7). When the shoots from such plants were subjected to osmotic stress induced by 5–20% polyethylene glycol, the transgenic leaves were more tolerant to PEG-induced osmotic stress in leaf disk assays (Figure 54.8a). While transgenic shoots recovered rapidly from PEG-induced osmotic stress, the untransformed control failed to do so.

Figure 54.7 Transgenic plants growing under contained poly house conditions.

Furthermore, when the PEG-stressed shoots were analyzed for stress tolerance, they showed increased accumulation of osmolytes such as raffinose and sucrose compared to untransformed control (Figure 54.8b).

These findings opened up a whole new way of developing tea plants with improved traits for better yield and quality. This is important because it will be now possible to utilize the transgenic technology for tea crop improvement, particularly with respect to drought, pests, and blister blight disease. Transgenic technology can also be used effectively for combating major reductions in yield due to winter dormancy, a problem experienced by many tea growing countries of the world.

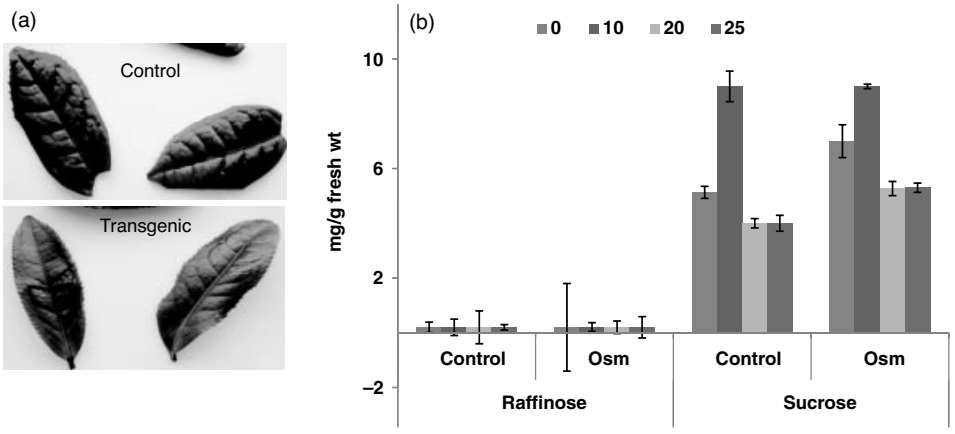

Figure 54.8 Response of untransformed control and transgenic plants to osmotic stress: (a) senescence of leaves at 20% PEG, (b) accumulation of osmolytes in response to increasing concentrations of PEG.

54.3 Areas for Future Research

Several books such as those by Barua [1], Wilson and Clifford [113], and Jain [2] combined together provided a global account on tea science. Palni *et al*. [114] reviewed various aspects of tea biotechnology covering *in vitro* propagation and its application, genetic transformation, artificial seeds, secondary metabolism, and biofertilizers. However, given the recent developments in the area of genomics, metabolomics, proteomics, and systems biology, tea needs enormous attention since research on this crop is still in its infancy.

1) Dormancy accompanies reduced rate of cell division and cell growth while dormancy release and vegetative bud growth is associated with the action of specific hormones and often accompanied by increased cell division [115–118]. Therefore, emphasis may be laid upon to understand the phenomenon of winter dormancy *vis-à-vis* cell cycle. For example, cell division is represented by a continuous cycle of "phases" G1, S, G2, and M phase. In dormant bud of potato, the cells are arrested in the G1 phase before the S phase of the cell cycle [116]. During dormancy release, upregulation of genes, such as D-type cyclins (CYCD) and histones [115, 117, 118], has been reported. From S phase, cell division proceeds to G2-M phase, which requires induction of the B-type cyclins (CYCB) and the cyclin-dependent kinase B (CDKB) gene [119].

   The plant hormones, auxin, cytokinin, and gibberellic acid, were implicated in expression and/or stability of CYCB and CDKB [120]. CYCB interacts with CDKB and initiates phosphorylation, activation of proteins, and expression of genes required for cytokinesis [121]. Abscisic acid induces expression of the p27CIP/KIP orthologue ICK1, an inhibitor of CDK action at the G1-S-phase transition [122]. Commitment to reenter the cell cycle is usually regulated at the G1-phase restriction point and is linked to protein kinase phosphorylation/dephosphorylation cascades, which are in turn activated by plasma membrane signaling machinery responsive to environmental or hormonal cues [123].
2) Another potent area would be to understand DNA methylation changes during winter dormancy. Along with the changes in gene expression, there are evidences for more general epigenetic changes associated with induction and release of endodormancy. Changes in DNA methylation and multiacetylation of histone have been observed during the induction and breaking of dormancy in potato buds [124, 125]. Increased DNA methylation following induction of dormancy suggested that chromatin remodeling might play a role in regulating bud dormancy. An intriguing observation was the strong induction of an aspen homologue of *FERTILISATION INDEPENDENT ENDOSPERM* (*FIE*), a polycomb family protein, during cambial dormancy [28]. *FIE* is believed to act as part of a complex that silences the transcription of genes necessary for proliferation through modification of chromatin structure [126, 127].
3) Yet another area would be combining kinematic approach [128] with transcriptomic, metabolomic, and proteomic experiments to unravel the important

processes such as mechanism of root growth under water stress [129]. For example, in soybean, three contiguous regions with distinct elongation characteristics can be identified in roots: (1) those where the elongation is maintained even under sever water stress (apical region), (2) the region where elongation takes place maximally under unstressed conditions but progressively inhibited under water stress, and (3) the regions where the growth decelerated in well-watered roots and was completely inhibited under water stress [130]. In such a situation, rather analyzing the whole root, which would give an average of event, it was essential to focus zone wise and analyze the proteome and transcriptome response. Indeed, the data showed differential proteome composition at these sites; enzymes related to isoflavonoid biosynthesis showed increased abundance in apical region, which was also positively correlated with increase in isoflavonoid content that possibly contributed to growth maintenance. Interestingly, region 2 was characterized by upregulation of caffeoyl-CoA *O*-methyltransferase, which is involved in lignin synthesis. Enhanced accumulation of lignin would lead to the inhibition of growth in this region. Similarly, Spollen *et al*. [131] studied transcriptome changes in these regions in maize tissue, wherein an increased expression of transcripts both for wall-loosening proteins in apical regions and for elements of ABA and ethylene signaling was evident under water stress.

4) Systems biology is yet another emerging approach to tackle various processes in tea. In a recent systems biology approach to unravel the underlying molecular program of apical bud development in poplar, combined transcript and metabolite profiling has been applied to a high-resolution time course from SD induction to dormancy [132]. Analysis of metabolite and gene expression dynamics allowed to reconstruct the temporal sequence of events during bud development. Importantly, to each of the following processes, bud formation, acclimation to dehydration and cold, and dormancy, specific sets of regulatory and marker genes and metabolites have been associated, which could provide a reference frame for future functional studies and for genetic approaches to assess adaptation of trees to climate change. Interestingly, the identification of a large set of genes commonly expressed during the growth-to-dormancy transitions in poplar apical buds, cambium, or *Arabidopsis* seeds suggested parallels in the underlying molecular mechanisms in different plant organs [133].

   Availability of high-throughput sequencing platforms by Solexa (Illumina, USA), 454 (Roche, USA), and SOLiD (Applied Biosystems, USA) offers opportunities to understand transcript/pathway shift in response to environmental cues. Novel genes and the pathways thus generated might help to improve the genetic potential of tea, particularly under the stressfull environment of drought, cold, or waterlogging. Even it would be important to decipher the performance of tea, both qualitatively and quantitatively, under the climate change scenario.

5) Deciphering the role of microRNA (miRNA) under abiotic stress could be yet another potent approach to decipher and improve tolerance in tea to abiotic

stress. MicroRNAs have been shown to participate in several plant processes including development, hormonal regulation, and response to environmental cues [133]. For example, miR159 was identified as a potent stress-responsive miRNA, and it also regulated the expression of transcription factors AtMYB33 and AtMYB101 [134]. Another stress-responsive and well-studied miRNAs were miR396 [135] and miR169 [136]. Tea offers opportunity to discover novel miRNAs that might be useful not only for tea but also for other crops.

6) Recent advances in DNA sequencing technologies have enriched genome-wide microsatellite and SNP markers. High-density SNP genotyping increased interest in genome-wide association (GWA) studies for the dissection of complex genetic traits in a number of crop plants including outbreed crops. Recent AFLP fingerprinting of 1644 tea accessions has led to identification of a set of core collections in Indian tea germplasm. Furthermore, sequencing of core collections with high-throughput next-generation sequencing will help generation of high-density genome-wide SNP data and will enable genome-wide association studies in tea for important traits including drought tolerance.

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References

1 Barua, D.N. (1989) *Science and Practice in Tea Culture*, Tea Research Association, Calcutta.

2 Jain, N.K. (ed.) (1999) *Global Advances in Tea Science*, Aravali Books International Pvt. Ltd., New Delhi.

3 FAO (2011) www.faostat.fao.org (Accessed 2 February, 2011).

4 Wight, W. (1962) Tea classification revised. *Curr. Sci.*, **31**, 298–299.

5 Chopra, D. and David, S. (2000) *The Chopra Centre Herbal Handbook*, Three Rivers Press, USA.

6 Jankun, J., Selman, S.H., Swiercz, R., and Skrzypczak-Jankun, E. (1997) Why drinking green tea could prevent cancer. *Nature*, **387** (6633), 561.

7 Koketsu, M. (1997) Antioxidative activity of tea polyphenols, in *Chemistry and Applications of Green Tea* (eds T. Yamamoto, L.R. Luneja, D.C. Chu, and D.C. Kim), CRC Press, Boca Raton, pp. 37–50.

8 Sakanaka, S., Kim, M., Taniguchi, M., and Yamamoto, T. (1989) Antibacterial substances in Japanese green tea extract against *Streptococcus mutan*: a carcinogenic bacterium. *Agric. Biol. Chem.*, **53** (9), 2307–2311.

9 Thiagarajan, G., Chandani, S., Sundari, C.S., Rao, S.H., Kulkarni, A.V., and Balasubramanian, D. (2001) Antioxidant properties of green and black tea, and their potential ability to retard the progression of eye lens cataract. *Exp. Eye Res.*, **73** (3), 393–401.

10 McKay, D.L. and Blumberg, J.B. (2002) The role of tea in human health: an update. *J. Am. Coll. Nutr.*, **21** (1), 1–13.

11 Carr, M.K.V. and Stephens, W. (1992) Climate, weather and the yield of tea, in *Tea: Cultivation to Consumption* (eds K.C. Willson and M.N. Clifford), Chapman & Hall, London, pp. 87–135.

12 Rohde, A. and Bhalerao, R.P. (2007) Plant dormancy in the perennial context. *Trends Plant Sci.*, **12** (5), 217–223.

13 Nandi, S.K., Palni, L.M.S., and Rashmi (1995) Chemical manipulation of dormancy in tea shoots and associated biochemical changes. *J. Plant. Crops.*, **23** (1), 52–58.

14 Nagar, P.K. (1996) Changes in endogenous abscisic acid and phenols during winter dormancy in tea (*Camellia sinensis* (L.) O. Kuntze). *Acta Physiol. Plant.*, **18** (1), 33–38.

15 Nagar, P.K. and Kumar, A. (2000) Changes in endogenous gibberellin activity during winter dormancy in tea (*Camellia sinensis* (L.) O. Kuntze). *Acta Physiol. Plant.*, **22** (4), 439–443.

16 Pietrini, F., Ianneli, M.A., Battistelli, A., Moscatello, S., Loreto, F., and Massacci, A. (1999) Effects on photosynthesis, carbohydrate accumulation and regrowth increase in maize genotypes with different sensitivity to low temperature. *Aust. J. Plant Physiol.*, **26** (4), 367–373.

17 Samis, K., Bowley, S., and McKersie, B. (2002) Pyramiding Mn-superoxide dismutase transgenes to improve persistence and biomass production in alfalfa. *J. Exp. Bot.*, **53** (372), 1343–1350.

18 Tang, W., Charles, T.M., and Newton, R.J. (2005) Overexpression of the pepper transcription factor CaPF1 in transgenic Virginia pine (*Pinus virginiana* Mill.) confers multiple stress tolerance and enhances organ growth. *Plant Mol. Biol.*, **59** (4), 603–617.

19 Vyas, D., Kumar, S., and Ahuja, P.S. (2007) Tea (*Camellia sinensis*) clones with shorter periods of winter dormancy exhibit lower accumulation of reactive oxygen species. *Tree Physiol.*, **27** (9), 1253–1259.

20 Vyas, D. and Kumar, S. (2005) Tea (*Camellia sinensis* (L.) O. Kuntze) clone with lower period of winter dormancy exhibits lesser cellular damage in response to low temperature. *Plant Physiol. Biochem.*, **43** (4), 383–388.

21 Vyas, D. and Kumar, S. (2005) Purification and partial characterization of a low temperature responsive Mn-SOD from tea (*Camellia sinensis* (L.) O. Kuntze.). *Biochem. Biophys. Res. Commun.*, **329** (3), 831–838.

22 Bezbaruah, H.P. (1971) Cytogenetical investigations of the family Theaceae; chromosome numbers in some *Camellia* species and allied genera. *Caryologia*, **24**, 421–426.

23 Tanaka, J., Taniguchi, F., Hirai, N., and Yamaguchi, S. (2006) Estimation of the genome size of tea (*Camellia sinensis*), camellia (*C. japonica*), and their interspecific hybrids by flow cytometry. *Tea Res. Rep.*, **101**, 1–7.

24 The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408** (6814), 796–815.

25 Zhou, S., Bechner, M.C., Place, M., Churas, C.P., Pape, L., Leong, S.A., Runnheim, R., Forrest, D.K., Goldstein, S., Livny, M., and Schwartz, D.C. (2007) Validation of rice genome sequence by optical mapping. *BMC Genomics*, **8**, 278.

26 Park, J.-S., Kim, J.-B., Hahn, B.-S., Kim, K.-H., Ha, S.-H., Kim, J.-B., and Kim, Y.-H. (2004) EST analysis of genes involved in secondary metabolism in *Camellia sinensis* (tea), using suppression subtractive hybridization. *Plant Sci.*, **166** (4), 953–961.

27 Chen, L., Zhao, L.P., and Gao, Q.K. (2005) Generation and analysis of expressed sequence tags from the tender shoots cDNA library of tea plant (*Camellia sinensis*). *Plant Sci.*, **168** (2), 359–363.

28 Schrader, J., Moyle, R., Bhalerao, R., Hertzberg, M., Lundeberg, J., Nilsson, P., and Bhalerao, R.P. (2004) Cambial meristem dormancy in trees involves extensive remodeling of the transcriptome. *Plant J.*, **40** (2), 173–187.

29 Derory, J., Léger, P., Garcia, V., Schaeffer, J., Hauser, M.-T., Salin, F., Luschnig, C., Plomion, C., Glössl, J., and Kremer, A. (2006) Transcriptome analysis of bud burst in sessile oak (*Quercus petraea*). *New Phytol.*, **170** (4), 723–738.

30 Mazzitelli, L., Hancock, R.D., Haupt, S., Walker, P.G., Pont, S.D.A., McNicol., J., Cardle, L., Morris, J., Viola, R., and Brennan, R. (2007) Co-ordinated gene expression during phases of dormancy release in raspberry (*Rubus idaeus* L.) buds. *J. Exp. Bot.*, **58** (5), 1035–1045.

31 Chakraborty, U., Dutta, S., and Chakraborty, B.N. (2002) Response of tea plants to water stress. *Biol. Plant*, **45** (4), 557–562.

32 Cheruiyot, E.K., Mumera, L.M., Ng'etich, W.K., Hassanali, A., and Wachira, F.N. (2010) High fertilizer rates increase susceptibility of tea to water stress. *J. Plant Nutr.*, **33** (1), 115–129.

33 Handique, A.C. and Manivel, L. (1986) Shoot water potential in tea II. Screening Tocklai cultivars for drought tolerance. *Two and a Bud*, **33**, 39–42.

34 Satyanarayana, N. and Cox., S. (1994) Factors influencing productivity of tea in drought. *J. Plant. Crops*, **22**, 87–92.

35 Marimuthu, S. and Kumar, R.R. (1998) Drought management in tea: a physiological approach. *Bull. UPASI Tea Sci. Depart.*, **51**, 16–18.

36 Kigalu, J.M. (2007) Effects of planting density and drought on the productivity of tea (*Camellia sinensis* L.) clones I. Measurement of water use in young tea using sap flow meters with a stem heat balance method. *Agr. Water Manage.*, **90** (3), 224–232.

37 Burgess, P.J. and Carr, M.K.V. (1993) Response of tea (*Camellia sinensis*) clones to drought. I. Yield, dry matter production and partitioning. *Asp. Appl. Biol.*, **34**, 249–258.

38 Rajasekar, R., Cox, S., and Satyanarayana, N. (1988) Evaluation of certain morphological and physiological factors in tea (*Camellia* L. spp.) cultivars under water stress. *J. Plantat. Crops*, **18**, 83–92.

39 Singh, I.D. and Handique, A.C. (1993) Breeding for resistance to water stress in tea (*Camellia sinensis* L.). *Two and a Bud*, **40**, 41–48.

40 Puthur, J.T., Sharmila, P., Prasad, K.V.S.K., and Saradhi, P.P. (1996) Proline overproduction: a means to improve stress tolerance in crop plants. *Botanica*, **46**, 163–169.

41 Matysik, J., Alia, B.B., and Mohanty, P. (2002) Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Curr. Sci.*, **82** (5), 525–531.

42 Wijeratne, M.A. (1996) Vulnerability of Sri Lankan Tea plantations to global climate change. *Water Air Soil Poll.*, **92** (1–2), 87–94.

43 Kamunya, S.M., Wachira, F.N., Owuor, P.O., Pathak, R.S., Wanyoko, J.K., Sharma, R.K., and Muoki, R.C. (2010) Quantitative genetic parameters for yield, drought tolerance and some quality traits in tea (*Camellia sinensis* L.) O. Kuntze. *Res. J. Agr. Sci.*, **1** (2), 53–65.

44 Ng'etich, W.K. and Stephens, W. (2001) Responses of tea to environment in Kenya. 1. Genotype×environment interactions for total dry matter production and yield. *Exp. Agr.*, **37** (3), 333–342.

45 Sharma, P. and Kumar, S. (2005) Differential display-mediated identification of three drought-responsive expressed sequence tags in tea (*Camellia sinensis* L.(O.) Kuntze). *J. Biosci.*, **30** (2), 231–235.

46 Carr, M.K.V. (2010) The role of water in the growth of the tea (*Camellia sinensis*) crop: a synthesis of research in Eastern Africa. 1. Water relations. *Exp. Agr.*, **46** (3), 327–349.

47 Netto, L.A., Jayaram, K.M., and Puthur, J.T. (2011) Clonal variation of tea [*Camellia sinensis* (L.) O. Kuntze] in countering water deficiency. *Phys. Mol. Biol. Plants*. doi: 10.1007/s12298-010-0040-8.

48 TRA (2011) www.tocklai.net (Accessed 2 February 2011).

49 UPASI (2011) www.upasitearesearch.org (Accessed 2 February 2011).

50 Bray, E.A. (1993) Molecular responses to water deficit. *Plant Physiol.*, **103** (4), 1035–1040.

51 Kreps, J.A., Wu, Y.J., Chang, H.S., Zhu, T., Wang, X., and Harper, J.F. (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol.*, **130** (4), 2129–2141.

52 Rana, N.K., Mohanpuria, P., and Yadav, S.K. (2008) Expression of tea cytosolic

glutamine synthetase is tissue specific and induced by cadmium and salt stress. *Biol. Plant*, **52** (2), 361–364.

53 Chen, Q., Yang, L., Ahmad, P., Wan, X., and Hu, X. (2010) Proteomic profiling and redox status alteration of recalcitrant tea (*Camellia sinensis*) seed in response to desiccation. *Planta*. doi: 10.1007/s00425-010-1322-7.

54 Singh, K., Rani, A., Kumar, S., Sood, P., Mahajan, M., Yadav, S.K., Singh, B., and Ahjua, P.S. (2008) An early gene of flavonoid pathway, flavanone 3-hydroxylase, exhibits a positive relationship with catechins content in tea (*Camellia sinensis* (L.) O. Kuntze). *Tree Physiol.*, **28** (9), 1349–1356.

55 Rani, A., Singh, K., Sood, P., Kumar, S., and Ahuja, P.S. (2009) *p-Coumarate: CoA ligase* as a key gene in the yield of catechins in tea [*Camellia sinensis* (L.) O. Kuntze]. *Funct. Integr. Genomics*, **9** (2), 271–275.

56 Singh, K., Kumar, S., Rani, A., Gulati, A., and Ahuja, P.S. (2009) *Phenylalanine ammonia-lyase* (*PAL*) and *cinnamate 4-hydroxylase* (*C4H*) and catechins (flavan 3-ols) accumulation in tea. *Funct. Integr. Genomics*, **9** (1), 125–134.

57 Singh, K., Kumar, S., Yadav, S.K., and Ahuja, P.S. (2009) Characterization of *dihydroflavonol 4-reductase* cDNA in tea [*Camellia sinensis* (L.) O. Kuntze]. *Plant Biotechnol. Rep.*, **3** (1), 95–101.

58 Singh, K., Rani, A., Paul, A., Dutt, S., Joshi, R., Gulati, A., Ahuja, P.S., and Kumar, S. (2009) Differential display mediated cloning of *anthocyanidin reductase* gene from tea (*Camellia sinensis*) and its relationship with the concentration of epicatechins. *Tree Physiol.*, **29** (6), 837–846.

59 Rani, A., Vats, S.K., Sharma, M., and Kumar, S., Catechin promotes root and shoot growth of *Arabidopsis thaliana* (Col-0) seedlings with concomitant changes in vascular system, IAA level and the net photosynthetic rate. *Bio. Plant.*, **55** (4): 779–782.

60 Tanksley, S.D. and McCouch, S.R. (1997) Seed banks and molecular maps: unlocking genetic potential from the wild. *Science*, **277** (5329), 1063–1066.

61 Plucknett, D.L., Smith, N.J.H., Williams, J.T., and Anishetty, N.M. (1983) Crop germplasm conservation and developing countries. *Science*, **220** (4593), 163–169.

62 Otieno, W., Sudoi, V., Wachira, F.N., Mamati, G.E., and Chalo, R. (2002) A report on outbreak of root knot nematodes on tea in Kerugoya and Imenti. *Tea Res. Found. Kenya Quarterly Bull.*, **7**, 6–8.

63 Tea Board of Kenya (2001) Annual Technical Report. Tea Research Foundation of Kenya, Tea Board of Kenya.

64 Bandyopadhyay, T. (2011) Molecular marker technology in genetic improvement of tea. *Int. J. Plant Breed. Genet.*, **5** (1), 23–33.

65 Raina, S.N., Ahuja, P. S. Sharma, R.K. *et al.* (2011) Genetic structure and diversity of India hybrid tea. *Genet. Resour. Crop Evol.* DOI 10.1007/s10722-011-9782-6.

66 Sharma, R.K., Negi, M.S., Sharma, S., Bhardwaj, P., Kumar, R., Bhattachrya, E., Tripathi, S.B., Vijayan, D., Baruah, A.R., Das, S.C., Bera, B., Rajkumar, R., Thomas, J., Sud, R.K., Muraleedharan, N., Hazarika, M., Lakshmikumaran, M., Raina, S.N., and Ahuja, P.S. (2010) AFLP-based genetic diversity assessment of commercially important tea germplasm in India. *Biochem. Genet.*, **48** (7–8), 549–564.

67 Freeman, S., West, J., James, C., Lea, V., and Mayes, S. (2004) Isolation and characterization of highly polymorphic microsatellites in tea (*Camellia sinensis*). *Mol. Ecol. Notes*, **4** (3), 324–326.

68 Hung, C.Y., Wang, K.H., Huang, C.C., Gong, C., Ge, X.J., and Chiang, T.Y. (2008) Isolation and characterization of 11 microsatellite loci from *Camellia sinensis* in Taiwan using PCR-based isolation of microsatellite arrays (PIMA). *Conserv. Genet.*, **9** (3), 779–781.

69 Ma, J.Q., Zhou, Y.H., Ma, C.L., Yao, M.Z., Jin, J.Q., Wang, X.C., and Chen, L. (2010) Identification and characterization of 74 novel polymorphic EST-SSR markers in

the tea plant, *Camellia sinensis* (Theaceae). *Am. J. Bot.* doi: 10. 3732/ajb. 1000376.

70 Sharma, R.K., Bhardwaj, P., Negi, R., Mohapatra, T., and Ahuja, P.S. (2009) Identification, characterization and utilization of unigene derived microsatellite markers in tea (*Camellia sinensis* L.). *BMC Plant Biol.*, **9**, 53.

71 Sharma, H., Kumar, R., Sharma, V., Kumar, V., Bhardwaj, P., Ahuja, P.S., and Sharma, R.K. (2011) Identification and cross transferability of 112 novel unigene derived microsatellite markers in tea (*Camellia sinensis* L). *Am. J. Bot.*, **98** (6), e133–e138.

72 Zhao, L.P., Liu, Z., Chen, L., Yao, M.Z., and Wang, X.C. (2008) Generation and characterization of 24 novel EST derived microsatellites from tea plant (*Camellia sinensis*) and cross-species amplification in its closely related species and varieties. *Conserv. Genet.*, **9** (5), 1327–1331.

73 Yang, J.B., Yang, J., Li, H.T., Zhao, Y., and Yang, S.X. (2009) Isolation and characterization of 15 microsatellite markers from wild tea plant (*Camellia taliensis*) using FIASCO method. *Conserv. Genet.*, **10** (5), 1621–1623.

74 Hackett, C.A., Wachira, F.N., Paul, S., Powell, W., and Waugh., R. (2000) Construction of a genetic linkage map for *Camellia sinensis* (tea). *Heredity*, **85** (4), 346–355.

75 Kamunya, S.M., Wachira, F.N., Pathak, R.S., Muoki, R.C., Wanyoko, J.K., Ronno, W.K., and Sharma, R.K. (2009) Quantitative genetic parameters in tea (*Camellia sinensis* (L.) O. Kuntze): I. Combining abilities for yield, drought tolerance and quality traits. *Afr. J. Plant Sci.*, **3** (5), 093–101.

76 Kamunya, S.M., Wachira, F.N., Pathak, R.S., Korir, R., Sharma, V., Kumar, R., Bhardwaj, P., Chalo, R., and Ahuja, P.S. (2010) Genomic mapping and testing for quantitative trait loci in tea (*Camellia sinensis* (L.) O. Kuntze). *Tree Genet. Genom.*, **6** (6), 915–929.

77 Kamunya, S.M. (2010) Genetic parameters and quantitative trait loci mapping in tea (*Camellia sinensis* (L.) O. Kuntze) loci) mapping in tea. Ph. D Thesis, Egerton University.

78 Michelmore, R.W., Paran, I., and Kesseli, R.V. (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA*, **88** (21), 9828–9832.

79 Bhattacharya, A. and Ahuja, P.S. (2001) Transgenic tea and its scope in tea crop improvement, in *Plant Genetic Engineering*, vol. 1 (eds R.P. Singh and P.K. Jaiwal), Sci-Tech Publishing Company, Houston, USA, pp. 115–130.

80 Kumar, N., Pandey, S., Bhattacharya, A., and Ahuja, P.S. (2004) Do leaf surface characteristics affect *Agrobacterium* infection in tea (*Camellia sinensis* (L.) O. Kuntze)? *J. Biosci.*, **29** (3), 309–317.

81 Liyuan, W., Hao, C., and Jian, Z. (2004) Advances on DNA molecular markers and gene-engineering in tea plants. *J. Tea Sci.*, **24** (1), 12–17.

82 Lopez, S.J., Kumar, R.R., Pius, P.K., and Muraleedharan, N. (2004) *Agrobacterium tumefaciens*-mediated genetic transformation in tea (*Camellia sinensis* (L.) O. Kuntze). *Plant Mol. Biol. Rep.*, **22** (2), 201a–202j.

83 Luo, Y.Y. and Liang, Y.R. (2000) Study on the construction of *Bt* gene expression vector and its transformation in tea plant. *J. Tea Sci.*, **20** (2), 141–147.

84 Matsumoto, S. and Fukai, M. (1998) *Agrobacterium tumefaciens* mediated gene transfer in tea plant (*Camellia sinensis*) cells. *Jpn. Agr. Res. Q.*, **32**, 287–291.

85 Matsumoto, S. and Fukai, M. (1999) Effect of acetosyringone application on *Agrobacterium* mediated gene transfer in tea plant (*Camellia sinensis*). *Bull.Natl. Res. Inst. Veg. Orna. Plants Tea, Shizuoka, Japan*, **14**, 9–15.

86 Biao, X., Toru, K., Jian, X., and Yongyan, B. (1998) Effect of polyphenol compounds in tea transformations. *Am. Soc. Plant Physiol.* Abstr. No. 314. http://abstracts.aspb.org/pb1998/public/P39/0310.shtml.

87 Wu, S., Liang, Y.R., Lu, J.L., Kim, H., and Wu, Y. (2003) Optimization of *Agrobacterium*-mediated and particle bombardment-mediated transformation

systems in tea plant (*Camellia sinensis*). *J. Tea Sci.*, **23** (1), 6–10.

88 Wu, S., Liang, Y.R., Lu, J.L., and Li, H.Y. (2005) Combination of particle bombardment-mediated and *Agrobacterium*-mediated transformation methods in tea plant. *J. Tea Sci.*, **25** (4), 255–264.

89 Siswanto, S.D. and Chaidamsari, T. (1999) Transient GUS expression and callus development of cocoa, coffee and tea following *Agrobacterium*-mediated transformation. *Menara Perkebunan*, **67** (2), 8–16.

90 Mondal, T.K., Bhattacharya, A., Sood, A., and Ahuja, P.S. (1999) An efficient protocol for somatic embryogenesis and its use in developing transgenic tea (*Camellia sinensis* (L.) O. Kuntze) for field transfer, in *Plant Biotechnology and In Vitro Biology in the 21st Century* (eds A. Altman, M. Ziv, and S.E. Izhar), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 181–184.

91 Mondal, T.K., Bhattacharya, A., and Ahuja, P.S. (2001) Induction of synchronous secondary somatic embryogenesis in *Camellia sinensis*. *J. Plant Physiol.*, **158** (7), 945–951.

92 Sandal, I., Bhattacharya, A., Ravindranath, S.D., Gulati, A., and Ahuja, P.S. (2006) Transgenic tea through biolistic using leaf explants. US Patent 712,939, filed Oct. 31, 2006.

93 Mondal, T.K., Bhattacharya, A., Ahuja, P.S., and Chand, P.K. (2001) Transgenic tea (*Camellia sinensis* (L.) O. Kuntze cv. Kangra jat) plants obtained by *Agrobacterium* mediated transformation of somatic embryos. *Plant Cell Rep.*, **20** (8), 712–720.

94 Mondal, T.K., Bhattacharya, A., Sood, A., and Ahuja, P.S. (2002) Factors affecting germination and conversion frequency of somatic embryos of tea (*Camellia sinensis* (L.) O. Kuntze). *J. Plant Physiol.*, **159** (12), 1317–1321.

95 Sandal, I., Kumar, A., Bhattacharya, A., Sharma, M., Shanker, A., and Ahuja, P.S. (2005) Gradual depletion of 2,4-D in the culture medium for indirect shoot regeneration from leaf explants of *Camellia sinensis* (L.) O. Kuntze. *Plant Growth Regul.*, **47** (2–3), 121–127.

96 Sandal, I., Bhattacharya, A., Ravindranath, S.D., Gulati, A., and Ahuja, P.S. (2006) Efficient method of preventing growth of microbial genetic transformant after transformation. US Patent 7145058 filed Dec. 5, 2006.

97 Sandal, I., Kaundal, A., Bhattacharya, A., Ravindranath, S.D., Gulati, A., and Ahuja, P.S. (2009) Substitute of acetosyringone: a thermolabile caffeine fraction of tea leaves. US Patent, 7608288. filed Oct. 27, 2007.

98 Saini, U. (2008) Development of transgenics for osmotin and *chitinase* or *chi* 11 genes in *Camellia sinensis* (L.) O. Kuntze. Ph.D. Thesis, Guru Nanak Dev Univ., Amritsar, India.

99 Bhattacharya *et al.* (2006) *Seed Sci. Res.*, **16** (3), 203–211.

100 Bonett, D. and McCourt, P. (1998) Genetic analysis of ABA signal transduction pathways. *Trends Plant Sci.*, **3** (6), 231–235.

101 Aberlenc-Bertossi, F., Chabrillange, N., Corbineau, F., and Duval, Y. (2003) Acquisition of desiccation tolerance in developing oil palm (*Elaeis guineensis* Jacq.) embryos *in planta* and *in vitro* in relation to sugar content. *Seed Sci. Res.*, **13** (2), 179–186.

102 Finch-Savage, W.E., Grange, R.I., Hendry, G.A.F., and Atherton, N.M. (1993) Embryo water status and loss of viability during desiccation in the recalcitrant species *Quercus robur* L, in *Proceedings of the Fourth International Workshop on Seeds: Basic and Applied Aspects of Seed Biology* (eds D. Côme and F. Corbineau), ASFIS, Paris, pp. 723–730.

103 Manfre, A.J., LaHatte, G.A., Climer, C.R., and Marcotte, W.R. (2009) Seed dehydration and the establishment of desiccation tolerance during seed maturation is altered in the *Arabidopsis thaliana* mutant *atem6-1*. *Plant Cell Physiol.*, **50** (2), 243–253.

104 Bhattacharya, A., Nagar, P.K., and Ahuja, P.S. (2002) Seed development in (*Camellia sinensis* (L.) O. Kuntze). *Seed Sci. Res.*, **12** (1), 39–46.

105 Sharma, P., Pandey, S., Bhattacharya, A., Nagar, P.K., and Ahuja, P.S. (2004) ABA associated biochemical changes during

somatic embryo development in *Camellia sinensis* (L.) (O.) Kuntze. *J. Plant Physiol.*, **161** (11), 1269–1276.

106 Sharma, P. (2003) Desiccation tolerance studies in tea (*Camellia sinensis* L. O. Kuntze) somatic embryos. M.Sc. Thesis, CSK HP Krishi Vishwavidyalay, Palampur, Himachal Pradesh.

107 Chen, Y. and Burris, J.S. (1990) Role of carbohydrates in desiccation tolerance and membrane behavior in maturing maize seed. *Crop Sci.*, **30** (5), 971–975.

108 Leprince, O., Bronchart, R., and Deltour, R. (1990) Changes in starch and soluble sugars in relation to the acquisition of desiccation tolerance during maturation of *Brassica campestris* seed. *Plant Cell Environ.*, **13** (6), 539–546.

109 Corbineau, F., Picard, M.A., Fougereux, J.A., Ladonne, F., and Côme, D. (2000) Effects of dehydration conditions on desiccation tolerance of developing pea seeds as related to oligosaccharide content and cell membrane properties. *Seed Sci. Res.*, **10** (3), 329–339.

110 Bhattacharya, A., Saini, U., Sharma, P., Nagar, P.K., and Ahuja, P.S. (2006) Osmotin-regulated reserve accumulation and germination in genetically transformed tea somatic embryos: a step towards regulation of stress tolerance and seed recalcitrance. *Seed Sci. Res.*, **16** (3), 203–211.

111 Kononowicz, A.K., Nelson, D.E., Singh, N.K., Hasegawa, P.M., and Bressan, R.A. (1992) Regulation of the *osmotin* gene promoter. *Plant Cell*, **4** (5), 513–524.

112 Sadat Noori, S.A. and Sokhansanj, A. (2008) Wheat plants containing an *Osmotin* gene show enhanced ability to produce roots at high NaCl concentration. *Russ. J. Plant Physiol.*, **55** (2), 256–258.

113 Wilson, K.C. and Cliford, M.N. (eds) (2002) *Tea: Cultivation to Consumption*, Chapman & Hall, London.

114 Palni, L.M.S., Hao, C., and Nakamura, Y. (1999) Advances in tea biotechnology, in *Global Advances in Tea Science* (ed. N.K. Jain,) Aravalli Books International Pvt. Ltd., New Delhi, pp. 449–462.

115 Devitt, M.L. and Stafstrom, J.P. (1995) Cell cycle regulation during growth-dormancy cycles in pea axillary buds. *Plant Mol. Biol.*, **29** (2), 255–265.

116 Campbell, M.A., Suttle, J.C., and Sell, T.W. (1996) Changes in cell cycle status and expression of $p34^{cdc2}$ kinase during potato tuber meristem dormancy. *Physiol. Plant*, **98** (4), 743–752.

117 Horvath, D.P., Chao, W.S., and Anderson, J.V. (2002) Molecular analysis of signals controlling dormancy and growth in underground adventitious buds of leafy spurge. *Plant Physiol.*, **128** (4), 1439–1446.

118 Freeman, D., Riou-Khamlichi, C., Oakenfull, E.A., and Murray, J.A.H. (2003) Isolation, characterization and expression of cyclin and cyclin-dependent kinase genes in Jerusalem artichoke (*Helianthus tuberosus* L.). *J. Exp. Bot.*, **54** (381), 303–308.

119 Dewitte, W. and Murray, J.A.H. (2003) The plant cell cycle. *Annu. Rev. Plant Biol.*, **54** (1), 235–264.

120 Mironov, V., Veylder, L.D., Montagu, M.V., and Inzé, D. (1999) Cyclin-dependent kinases and cell division in plants: the nexus. *Plant Cell*, **11** (4), 509–521.

121 Francis, D. and Sorrell, D.A. (2001) The interface between the cell cycle and plant growth regulators: a mini review. *Plant Growth Regul.*, **33** (1), 1–12.

122 Wang, H., Qi, Q., Schorr, P., Cutler, A.J., Crosby, W.L., and Fowke, L.C. (1998) ICK1, a cyclin dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. *Plant J.*, **15** (4), 501–510.

123 Anderson, J.V., Chao, W.S., and Horvath, D.P. (2001) A current review on the regulation of dormancy in vegetative buds. *Weed Sci.*, **49** (5), 581–589.

124 Law, R.D. and Suttle, J.C. (2003) Transient decreases in methylation at 5′-CCGG-3′ sequences in potato (*Solanum tuberosum* L.) meristem DNA during progression of tubers through dormancy precede the resumption of sprout growth. *Plant Mol. Biol.*, **51** (3), 437–447.

125 Law, R.D. and Suttle, J.C. (2004) Changes in histone H3 and H4 multi-acetylation during natural and forced dormancy

break in potato tubers. *Physiol. Plant.*, **120** (4), 642–649.

126 Grossniklaus, U., Spillane, C., Page, D.R., and Köhler, C. (2001) Genomic imprinting and seed development: endosperm development with and without sex. *Curr. Opin. Plant Biol.*, **4** (1), 21–27.

127 Katz, A., Oliva, M., Mosquna, A., Hakim, O., and Ohad, N. (2004) FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. *Plant J.*, **37** (5), 707–719.

128 Erickson, R.O. and Silk, W.K. (1980) The kinematics of plant growth. *Sci. Am.*, **242**, 134–151.

129 Yamaguchi, M. and Sharp, R.E. (2010) Complexity and coordination of root growth at low water potentials: recent advances from transcriptomic and proteomic analyses. *Plant Cell Environ.*, **33** (4), 590–603.

130 Yamaguchi, M., Valliyodan, B., Zhang, J., Lenoble, M.E., Yu, O., Rogers, E.E., Nguyen, H.T., and Sharp, R.E. (2010) Regulation of growth response to water stress in the soybean primary root. I. Proteomic analysis reveals region-specific regulation of phenylpropanoid metabolism and control of free iron in the elongation zone. *Plant Cell Environ.*, **33** (2), 223–243.

131 Spollen, W.G., Tao, W., Valliyodan, B., Chen, K., Hejlek, L.G., Kim, J.-J., LeNoble, M.E., Zhu, J., Bohnert, H.J., Henderson, D., Schachtman, D.P., Davis, G.E., Springer, G.K., Sharp, R.E., and Nguyen, H.T. (2008) Spatial distribution of transcript changes in the maize primary root elongation zone at low water potential. *BMC Plant Biol.*, **8**, 32.

132 Ruttink, T., Arend, M., Morreel, K., Storme, V., Rombauts, S., Fromm, J., Bhalerao, R.P., Boerjan, W., and Rohde, A. (2007) A molecular timetable for apical bud formation and dormancy induction in poplar. *Plant Cell*, **19** (8), 2370–2390.

133 Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006) MicroRNAS and their regulatory roles in plants. *Annu. Rev. Plant Biol.*, **57**, 19–53.

134 Reyes, J.L. and Chua, N.H. (2007) ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. *Plant J.*, **49** (4), 592–606.

135 Liu, D., Song, Y., Chen, Z., and Yu, D. (2009) Ectopic expression of *miR396* suppresses GRF target gene expression and alters leaf growth in *Arabidopsis*. *Physiol. Plant.*, **136** (2), 223–236.

136 Zhu, J.K. (2008) Reconstituting plant miRNA biogenesis. *Proc. Natl. Acad. Sci. USA*, **105** (29), 9851–9852.

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