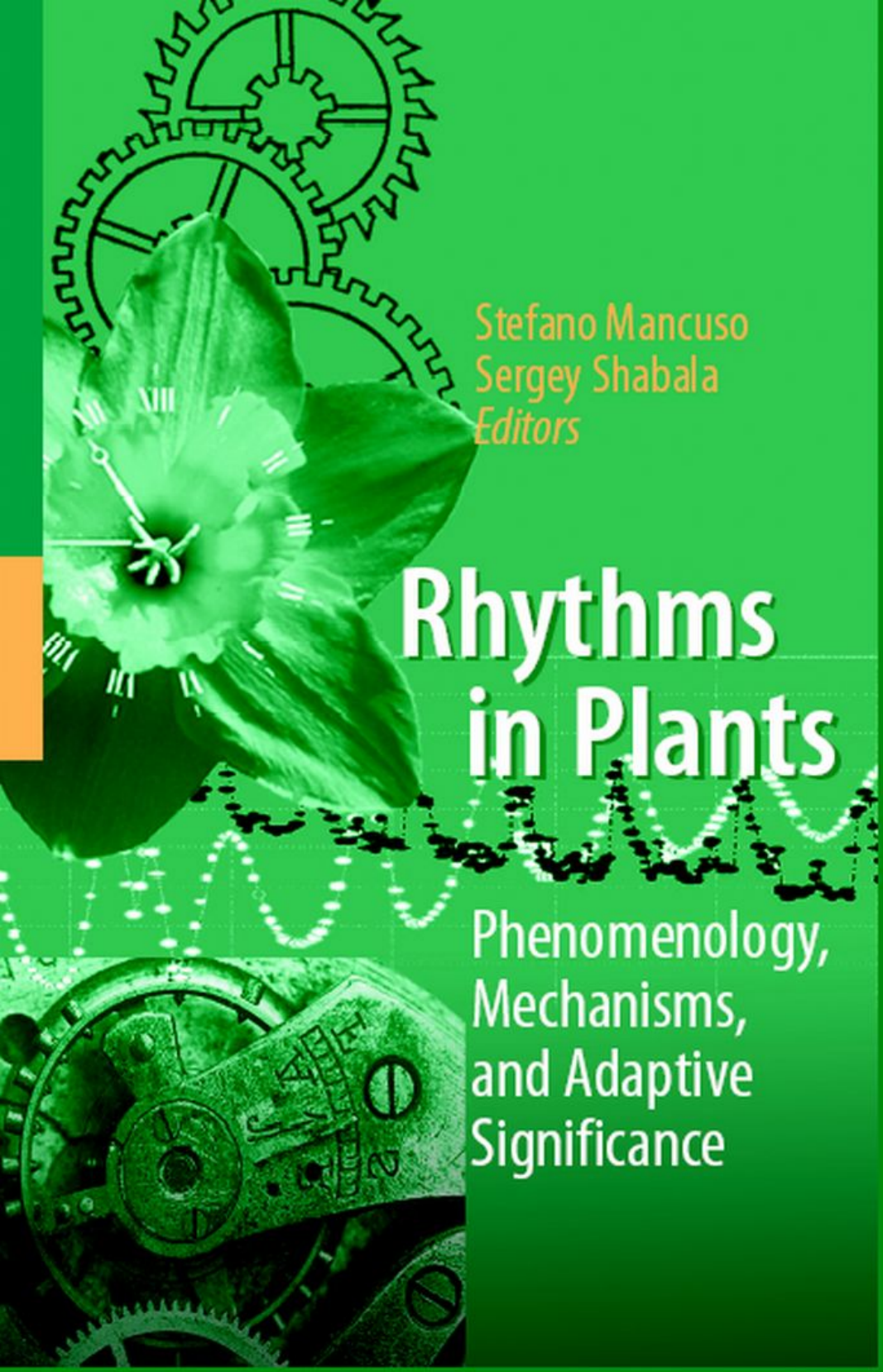

Stefano Mancuso
Sergey Shabala
Editors

Rhythms in Plants

Phenomenology, Mechanisms, and Adaptive Significance

Springer

Rhythms in Plants: Phenomenology, Mechanisms, and Adaptive Significance

S. Mancuso S. Shabala (Eds.)

Rhythms in Plants
Phenomenology, Mechanisms, and Adaptive Significance

With 84 Figures, 3 in Color, and 5 Tables

Springer

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Stefano Mancuso dedicates this volume to Professor Emeritus Franco Scaramuzzi on his 80th birthday in grateful and affectionate acknowledgement of his enthusiastic support as teacher, friend and colleague.

Preface

Rhythm is the basis of life, not steady forward progress. The forces of creation, destruction, and preservation have a whirling, dynamic interaction.
Kabbalah quote

Rhythmic phenomena are an omnipresent attribute of behavioural and physiological processes in biology. From cell division to flowering, clocklike rhythms pervade the activities of every physiological process in plants, often in tune with the day/night cycle of the earth.

Research into the rhythmic leaf movements in nyctinastic plants in the early 18th century provided the first clue that organisms have internal clocks. However, observations about rhythmic movement in plants had been discussed already in the pre-Christian era. As early as the 4th century B.C., Androsthenes, scribe to Alexander the Great, noted that the leaves of *Tamarindus indica* opened during the day and closed at night (Bretzl 1903).

Some early writers noticed single movements of parts of plants in a cursory manner. Albertus Magnus in the 13th century and Valerius Cordus in the 16th thought the daily periodical movements of the pinnate leaves of some *Leguminosae* worth recording (Albertus Magnus 1260; for Cordus 1544, see Sprague and Sprague 1939). John Ray, in his 'Historia Plantarum' towards the end of the 17th century (Ray 1686–1704), commences his general considerations on the nature of plants with a succinct account of *phytodynamical* phenomena, but does not clearly distinguish between movements stemming from irritability and those showing daily, periodical rhythms; the latter, he writes, occur not only in the leaves of *Leguminosae* but also in almost all similar pinnate leaves. In addition to these periodical movements of leaves, he reports the periodical opening and closing of the flowers of *Calendula*, *Convolvulus*, *Cichorium* and others.

In 1729, the French physicist Jean Jacques d'Ortous de Mairan discovered that mimosa plants kept in darkness continued to raise and lower their leaves with a ~24 h rhythm. He concluded that plants must contain some sort of internal control mechanism regulating when to open or close the leaves.

Carolus Linnaeus studied the periodical movements of flowers in 1751 and those of leaves in 1755, but offered no mechanical explanation (Linnaeus 1770). He contented himself with describing the external conditions of these phenomena in many species, classifying them and giving a new name – *sleep*

of plant – to those periodical movements observed at night, considering that the plants had then assumed a position of *sleep*. Indeed, he did not use the word at all in a metaphoric sense, for he saw in this sleep of plants a phenomenon entirely analogous to that in animals. It should also be mentioned that he stated correctly that the movements connected with the sleep of plants were not caused by changes in temperature but rather by change in light, since these took place at uniform temperature in a conservatory. Knowing that each species of flower has a unique time of day for opening and closing, Linnaeus designed a garden clock in which the hours were represented by different varieties of flowers. His work supported the idea that different species of organisms demonstrate unique rhythms.

Building on these classical findings, the last decades have experienced a period of unprecedented progress in the study of rhythmical phenomena in plants. Innovations in molecular biology, micro- and nanotechnology and applied mathematics (e.g. hidden patterns, chaos theory) are providing new tools for understanding how environmental signals and internal clocks regulate rhythmic gene expression and development. Needless to say, this fast, nearly astounding pace of discoveries shows how extremely this subject has changed, and this is well reflected in the various chapters of this book which covers aspects of plant physiology neither recognisable nor quantifiable only a few years ago.

The capacity to experience oscillations is a characteristic inherent to living organisms. Many rhythms, at different levels extending from the cell to the entire plant, persist even in complete isolation from major known environmental cycles. Actually, 24-h rhythms (circadian rhythms) are not the only biological rhythms detectable in plants – there are also those extending over longer periods (infradian rhythms), either a month, year or a number of years, as well as shorter rhythms (ultradian rhythms) lasting several hours, minutes, seconds, etc. Accordingly, natural rhythms can be considered to lie outside the periods of geophysical cycles. This means that living matter has its own time, i.e. the 'biological time' is a specific parameter of living functions which can not be neglected, as has often been the case in traditional plant biology.

Unlike circadian rhythms, ultradian rhythms have received little attention from plant biologists. Among the causes of this underestimation is the fact that ultradian rhythms are readily overlooked in experiments in which observations are made only intermittently, or are treated as unwanted noise. Classically, oscillations of data during discontinuous measurements are either ignored or attributed to sampling inaccuracy or error in the technique used, rather than to biological rhythmicity. In addition, the common practice of pooling and averaging data collected from different specimens will serve – given that no two specimens are likely to be completely in phase – to obscure rhythmicity. On the whole, modern plant biology is poorly equipped for the study of ultradian rhythms. These are best studied in single specimens, using high-resolution, non-invasive, uninterrupted recording techniques. Such a

holistic approach to physiology runs counter-current to the prevalent reductionism which emphasizes the use of averaged data collected by means of invasive measurements in as many samples as possible.

It must be noted that, since biological rhythms are genetically transmitted, these phenomena necessarily have an inherited character. Researchers are aware of the fact that plants live and act in time. Therefore, the concept of cyclic biological time is not entirely extraneous to scientific doctrine. Traditionally, however, plant biologists consider time as an implicit quantity, relegating it to a role of external factor.

It has been suggested that the gene inherits not only the capacity to clone but also the capacity to endure (*chronon*). The concept of chronon refers to the expression of genes as a function of chronological time. The concept of chronome relates to the expression of genes as a function of biological time, which is cyclical, irreversible and recursive. Accordingly, chronological time could be seen as the summation of iterated periods, which constitute the time base of biological rhythms.

The cycles of life are ultimately biochemical in mechanism but many of the principles which dominate their orchestration are essentially mathematical. Thus, the task of understanding the origins of rhythmic processes in plants, apart from numerous experimental questions, challenges theoretical problems at different levels, ranging from molecules to plant behaviour. The study of data on biological fluctuations can be the means of discovering the existence of underlying rhythms. It might be of interest, for example, to account for periodic variability in measurements of hormone concentrations, membrane transport rates, ion fluxes, protein production, etc. Nevertheless, before engaging in the necessary statistical processing for the detection of cycles in a system, it is essential to represent the system to be studied by means of a model: one that is explicative or one that is representative and predictive.

This volume concentrates on modelling approaches from the level of cells to the entire plant, focusing on phenomenological models and theoretical concepts. The book has been subdivided into four main parts, namely:

1. Physiological implications of oscillatory processes in plants;
2. Stomata oscillations;
3. Rhythms, clocks and development;
4. Theoretical aspects of rhythmical plant behaviour,

assembled for an intended audience composed of the large and heterogeneous group of science students and working scientists who must, due to the nature of their work, deal with the study and modelling of data originating from rhythmic systems in plants. Hopefully, the wide range of subjects will excite the interest of readers from many branches of science: physicists or chemists who wish to learn about rhythms in plant biology, and biologists who wish to learn how these rhythmic models are generated.

Finally, the Editors gratefully acknowledge the assistance of a number of people and institutions without whose help this project could not have been carried out. First of all, we are most deeply indebted to the contributors of the chapters presented here, whose enthusiasm and dedication have made this book a reality. We also acknowledge the *Fondazione Ente Cassa di Risparmio di Firenze* for financial support given to the LINV – Laboratorio Internazionale di Neurobiologia Vegetale, University of Firenze, as well as the Australian Research Council for supporting research on membrane transport oscillators at the University of Tasmania. Last but not least, we express our sincere appreciation to Dr. Andrea Schlitzberger and Dr. Christina Eckey, at Springer, for their guidance and assistance during the production of the book.

December 2006

Stefano Mancuso
Sergey Shabala

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Part 1
Physiological Implications of Oscillatory Processes in Plants

1 Rhythmic Leaf Movements: Physiological and Molecular Aspects

Nava Moran

Abstract

Daily periodic plant leaf movements, known since antiquity, are dramatic manifestations of "osmotic motors" regulated by the endogenous biological clock and by light, perceived by phytochrome and, possibly, by phototropins. Both the reversible movements and their regulation usually occur in specialized motor leaf organs, pulvini. The movements result from opposing volume changes in two oppositely positioned parts of the pulvinus. Water fluxes into the motor cells in the swelling part and out of the motor cells in the concomitantly shrinking part are powered by ion fluxes into and out of these cells, and all of these fluxes occur through tightly regulated membranal proteins: pumps, carriers, and ion and water channels. This chapter attempts to piece together those findings and insights about this mechanism which have accumulated during the past one and a half decades.

1.1 Introduction

1.1.1 Historical Perspective

Almost every text on chronobiology tells us that the ancients were already aware of the rhythmic movements of plants, and even relied on them in scheduling their prayers. The first documented experiment attempting to resolve if this rhythm was inherent to the plant, rather than being stimulated by sunlight, was that of the French astronomer, De Mairan. His sensitive plant (probably *Mimosa pudica*) continued moving its leaves even when kept in darkness (De Mairan 1729). Since De Mairan's days, and for over 2 centuries, leaf movements served as the sole indicators of the internal working of plants, and increasingly intricate designs were conceived for

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movement-monitoring devices (see also Nozue and Maloof 2006). During the 18th and the 19th centuries, experiments with the "sleep movements" of leaves (a name coined by Linnaeus) led to the gradual emergence of the concept of the osmotic motor (Pfeffer 1877), and of the concept of an internal oscillator – an endogenous biological clock – for which leaf movements serve as "clock hands". In the 20th century, biological clocks began to be studied also in animals. Beatrice Sweeney presented a detailed and vivid account of this conceptual evolution (Sweeney 1987).

Among the best studied rhythmic movements are those of the pulvini of the compound leaves of the legumes *Albizzia*, *Mimosa*, *Samanea*, *Robinia* and *Phaseolus*. While observing the "hands of the clock", investigators probed the internal mechanism, in an attempt to map the susceptibility of the oscillator and, thus, to deduce its chemical nature. They altered the illumination regimes, varied the light intensity and quality, and applied various pharmacological agents to the pulvinus (e.g. see the review by Satter and Galston 1981 and, more recently, work by Mayer et al. 1997, and Gomez et al. 1999). During the past few decades, an increasing arsenal of technological developments enabled more sophisticated measurements and monitoring of variables other than only leaf displacement. The forces involved in the movement have been determined (Gorton 1990; Irving et al. 1997; Koller 2001), immunohistochemistry has been applied (e.g. in the cellular immuno-gold localization of phytochrome, the photoreceptor affecting leaf movement; Moysset et al. 2001), the related distribution of various ions and other elements has been studied using ion-selective microelectrodes (e.g. Lee and Satter 1989; Lowen and Satter 1989), and X-ray microanalysis (e.g. Satter et al. 1982; Fromm and Eschrich 1988c; Moysset et al. 1991), patch-clamp and molecular biology analyses of pulvinar channels have begun (Moran et al. 1988; Stoeckel and Takeda 1993; Jaensch and Findlay 1998; Moshelion et al. 2002a, b).

Initial answers to the intriguing questions about how leaf movement is executed, and how the endogenous rhythm – and external signals, mainly light – affect the pulvinar "motor" have been collected in a small but thorough compendium on the pulvinus by Satter et al. (1990). During the following 16 years, these questions have been addressed with an increasing resolution, sometimes "borrowing" from the molecular insights developed in the much more numerous and extensive studies of stomatal guard cells (as in Fan et al. 2004). These later findings and insights into leaf movements are the main focus of this chapter.

1.1.2 The Types of Leaf Movements

Leaf movements can be repetitious and rhythmic, or provoked (Fig. 1.1). Stimulated movements can be classified according to their directionality: *tropic* movements are related to the direction of the stimulus which caused them whereas *nastic* movements are stimulus unrelated. Thus, leaf unfolding

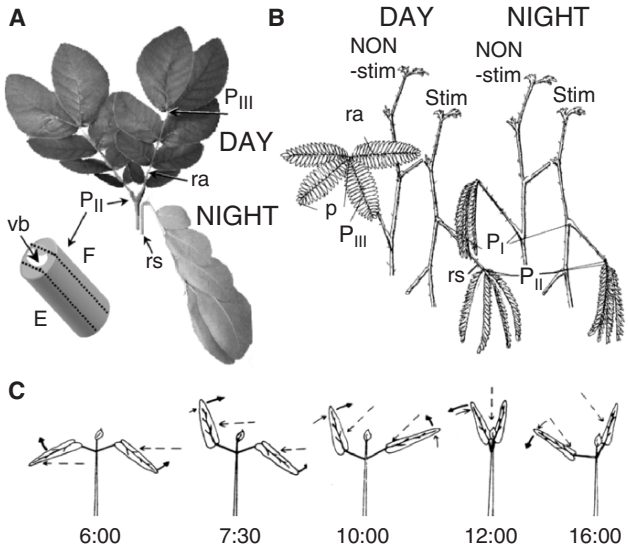

Fig. 1.1 Types of leaf movements. A Nyctinastic movements of the terminal pinnae of the compound leaf of *Samanea saman* (Jacq.) Merrill. *Insets* A schematic drawing of a pulvinus: *E* extensor, *F* flexor, *vb* vascular bundle, P_{II}, P_{III} secondary and tertiary pulvini, *ra* rachilla, *rs* rachis (reproduced with permission, Moshelion et al. 2002a). B Seismonastic and nyctinastic leaf movement of *Mimosa pudica* L.: *p* pinnae, P_I primary pulvinus; other abbreviations as in **A** (reproduced with permission, Fromm and Eschrich 1988b). C Primary (laminar) leaves of *Phaseolus vulgaris* L., showing paraheliotropism in the field (reproduced with permission, Berg 1986). Note the movement of the leaf blades (*arrows*), adjusting the angle of the incident light (*dashed arrows*) at the indicated hours. A "purely" nyctinastic movement in the laboratory would occur between a horizontal and a vertical-*down* position of both leaves (not shown)

in response to the turning on of diffuse light is *photo*nastic whereas leaf folding with the onset of darkness is *scoto*nastic; the turning of leaves towards directed light is termed *photo*tropic and, towards the sun, *helio*tropic (Fig. 1.1c). Movement in response to touch – such as the clasping of the Venus fly trap (*Dionaea muscipula*) leaf lobes when irritated by an insect, or the curling of a gently stroked pea tendril – is termed *thigmo*nastic; the folding down of the *Mimosa pudica* leaf upon shaking the plant is *seismo*nastic and, upon exposure to the heat of a flame, *thermo*nastic; the turning of leaves upwards after the shoot is placed horizontally is negatively *gravi*tropic. Frequently, leaves perform more than one type of movements, and different parts of a leaf can perform different types of movements. For example, the *Mimosa* primary pulvinus exhibits also nyctinasty, seismonasty and thigmonasty whereas the secondary pulvinus does not respond to seismonastic stimuli (Fig. 1.1b; Fromm and Eschrich 1988b). *Samanea* leaf movements are largely insensitive to touch and shaking.

Rhythmic leaf movements can be related to growth and be *non*-reversible, such as those of cotyledons of *Arabidopsis* seedlings or the leaves of growing tobacco plants. The *epi*nastic leaf movement of tobacco, for example, is based on alternating spurts of growth of the upper and lower leaf surface, and this uneven growth reveals a control by light and the circadian clock (Siefritz et al. 2004). Other examples can be found in a review by Wetherell (1990). While the tissue expansion likely occurs via a mechanism similar to that for pulvinar tissues (see below), the irreversibility of these growth processes is thought to be related to interstitial deposits in cell wall material and to decrease in wall extensibility (Wetherell 1990, and references therein).

Rhythmic leaf movements can be completely reversible, such as the *nycti*nastic movements of many legumes (*Samanea saman*, *Accacia lophanta*, *Albizzia julibrissin*, *Phaseolus vulgaris*, *Desmodium gyrans* and the abovementioned *Mimosa pudica*), and also of some plants of a few other families, e.g. wood sorrels (*Oxalidaceae*) and mallows (*Malvaceae*). These reversible movements originate in the pulvinus (Fig. 1.1), a mature, specialized motor organ at the leaf base, and their daily persistence is a manifestation of regulation by light and the *circa*dian clock. In the dark or under constant low-level illumination, the circadian rhythm displays its "free-running", genetically dictated periodicity which can range from roughly 20 to 29 h. Period length and its manifestation depend also on other factors. For example, in *Phaseolus coccineus*, the circadian laminar leaf movement started 9 days after sowing in soil. The period length decreased progressively with pulvinus maturation (from 31.3 to 28.6 h under constant illumination), and these periods became more than 1 h shorter when the leaves were cut off and watered via petioles (Mayer et al. 1999).

Normally, however, daily light resets the phase of the rhythm and adjusts it to a 24-h period. Rhythmic movements can additionally comprise one or more *ultra*dian rhythms (with significantly shorter periods – between tens of minutes to several hours; Millet et al. 1988; Engelmann and Antkowiak 1998; see also chapter [3] on ultradian rhythms).

Light has a profound effect on the rhythmic leaf movement, and it is also easily quantifiable. Therefore, this stimulus is very widely used to perturb leaf movement rhythms, to change their phase, and to alter their period. Changing these two rhythm properties is a key criterion for having affected the internal "oscillator". Red, far-red and blue light have different effects on the rhythm (reviewed by Satter and Galston 1981; Sweeney 1987).

Acute Versus Circadian It is important to note that the same stimuli evoke also short-lived, or acute, responses lasting for only one to a few periods following the stimulus. These transient responses are superimposed on ("mask") the responses attributable to changes in the clock (shifting the phase and changing the period length), which persist during many cycles. In the very schematic general portrayal of the system shown in Fig. 1.2, the clock-resetting stimulus acts along an input pathway to the clock, altering the

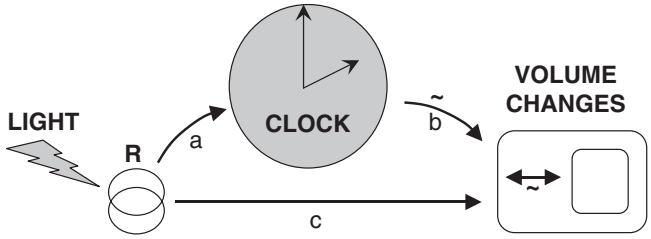

Fig. 1.2 Light stimulates cell volume changes. A model of clock-mediated (circadian) and clock-independent ("acute") pathways. *a* Light, perceived by one or more light receptor(s), R, affects the clock. *b* The clock governs volume changes, imparting fluctuations (~) in activity or in abundance to the pathway intermediates. *c* Light affects directly the volume changes (*bidirectional arrow*)

way the *clock* directs the osmotic motor of the leaf movement, while the acute stimulus bypasses the clock and acts directly on the osmotic motor. Employing "acute" stimuli in the study of the clock's role in regulating leaf movement is justified by the underlying assumptions (1) that the mechanism of the *execution* of the movements, i.e. of the volume and turgor changes, is identical for both types of movements, the stimulated and the rhythmic, and (2) that the *photoreceptors* in both pathways are identical (which, in plants, has not yet been disproved). Thus, both pathways are assumed to differ wholly, or partially, "only" in the transduction cascades, i.e. in the chemical reactions between light perception and the regulation of the transporters.

1.2 The Mechanism of Leaf Movement: the Osmotic Motor

1.2.1 Volume Changes

1.2.1.1 The Mechanics of Movement

Since the movement of a leaf or leaflet results from the changes in the shape of its subtending pulvinus, volume changes must occur anisotropically in the pulvinar tissues. Indeed, the pulvinar motor consists of two distinct, positionally and functionally opposed regions: an "extensor" – which extends longitudinally during leaf opening, and a "flexor", which appears contracting ("flexing") longitudinally at the same time. During leaf closure, the reverse changes occur. Radial inflexibility of the epidermis constrains these changes to the longitudinal axis but the flexibility of the vascular core, along with its inextendability, cause the curvature of the pulvinus without, in fact, affecting its length (Koller and Zamski 2002). It appears that extensors and flexors differ also in the extent of generating the movement-driving pressures. For example, in the *Phaseolus vulgaris* laminar pulvinus, the

excision of the flexor did not seem to alter any of the properties of the circadian leaf oscillation – period, phase and amplitude – whereas, when the major part of the extensor was cut away, the amplitude was greatly reduced (although the period and the phase of the leaf movements remained unchanged; Millet et al. 1989).

1.2.1.2 Volume Changes of Isolated Protoplasts

The turgor changes in the pulvinar motor tissues reflect the turgor changes of the individual motor cells and these, in turn, reflect the elastic properties of the cell walls, together with the volume changes. Confounding effects of the cell wall may be avoided if experiments are performed on protoplasts. Indeed, protoplasts appear to be an appropriate physiological system for studying the circadian rhythm of volume changes. Flexor protoplasts isolated from the bean (*P. coccineus*) laminar pulvini swelled and shrunk under continuous light for over 200 h with a 28-h period, resembling the period of the pulvinar cells in situ under similar conditions (Mayer and Fischer 1994). Extensor protoplasts seemed to exhibit the same rhythm and, curiously, they cycled with the same *phase* as the flexors, at least during the initial 70 h, as if their internal clock had shifted by 180° relative to their original in-situ rhythm. Nevertheless, the extensors could be entrained to a 24-h rhythm by cycles of 14 h light/10 h dark, this time shrinking "appropriately" in darkness (Mayer and Fischer 1994). Thus, the isolated pulvinar protoplasts seem to "remember" their origin and retain the physiological properties of their source tissues. Moreover, the motor cells of the pulvinus are themselves the site of the rhythm generator, containing both the "oscillator" and the "motor", as evident from the rhythmic volume changes of isolated protoplasts (in *Phaseolus*, Mayer and Fischer 1994, and also as shown for flexors of *Samanea* by Moran et al. 1996).

1.2.2 The Ionic Basis for the Osmotic Motor

1.2.2.1 The Current Model

The currently accepted model for the volume changes of pulvinar cells does not differ in principle from that accepted for the stomata guard cells, with an exception that in contrast to guard cells, in the intact pulvinus the solute and water fluxes may occur to some extent also via plasmodesmata interconnecting the pulvinar motor cells (Morse and Satter 1979; Satter et al. 1982).

In the swelling phase, an activated proton pump (a P-type H^+-ATPase) hyperpolarizes the cell, which creates the electrochemical gradient for the influx of K^+ via K channels (e.g., Kim et al. 1992, 1993) and the proton-motive

force for the uphill uptake of Cl^-, possibly via a proton-anion symporter (Satter et al. 1987), and which also open the gates of K^+-influx channels. Eventually, K^+ and Cl^- accumulate in the cell vacuole. In the absence of external Cl^-, the malate content of the swelling tissues increases (Mayer et al. 1987; Satter et al. 1987). Water, driven by the changing water potential difference across the cell membrane, increases the cell volume and turgor, entering the cells via the membrane matrix and via aquaporins.

In the shrinking phase, the proton pump halts and the motor cell depolarizes. Depolarization may be aided by passive influx of Ca^{2+} via Ca channels and passive efflux of Cl^- via anion channels. K^+-*in*flux channels close while K^+-release channels open. The electrochemical gradient now drives also K^+ efflux. Loss of solutes (KCl) drives water efflux via the membrane matrix and aquaporins. The volume and turgor of the motor cells decrease.

1.2.2.2 Membrane Potential

Changes in membrane potential provided early clues about the ionic basis of leaf movement. Racusen and Satter measured the membrane potential in *Samanea* flexors and extensors in whole, continuously darkened, secondary terminal pulvini impaled with microelectrodes, and found it to oscillate with a ca. 24-h rhythm between −85 and −40 mV (extensor) and between −100 and −35 mV (flexors), with the extensors "sinusoid" preceding that of the flexors by about 8 h (Racusen and Satter 1975). Membrane potential varied also in response to light signals which caused leaf movement (see Sect. 1.3.2.1 below, and Racusen and Satter 1975, and also Sect. 1.3.2.2). Later measurements of membrane potential, using a membrane-soluble fluorescent dye (3,3′-dipropylthiadicarbocyanine iodide, DiS-C3(5)), provided additional details about the translocation of ions (Kim et al. 1992, and see Sect. 1.2.3.4 below).

1.2.2.3 Mechanisms Underlying Volume Changes

Ions Involved in Leaf Movements Results of X-ray microanalysis suggest that the solute concentration changes are primarily those of potassium and chloride, consistent with the occurrence of their massive fluxes across the plasma membrane into the swelling cells and out of the shrinking cells (Satter and Galston 1974; Kiyosawa 1979; Satter et al. 1982; Gorton and Satter 1984; Moysset et al. 1991). At the same time, measurements with ion-sensitive electrodes enabled dynamic, real-time observations of changes in the apoplastic activity of protons (Lee and Satter 1989) and potassium ions (Lowen and Satter 1989; Starrach and Meyer 1989). Generally, proton and K^+ activities varied in opposite directions (see also Starrach and Meyer 1989 and references therein, and Lee 1990).

Non-Ionic Regulation Osmotically driven shrinking based on the efflux of ions normally suffices to explain volume changes on the scale of minutes. The puzzling rate of the seismonastic response of *Mimosa pudica* (leaflet folding on the scale of seconds) invited additional investigations. Thus, seismonastic stimulation of the leaf caused sudden unloading of ^{14}C-labelled sucrose from the phloem into the pulvinar apoplast in the primary pulvinus, lowering the water potential beneath that of the extensors and probably enhancing their shrinkage, leading to leaf closure within a few seconds. This was accompanied by a brief membrane depolarization of the sieve-element, recorded via an aphid stylet serving as an intracellular microelectrode. During re-swelling, the extensors accumulated the labelled material (Fromm and Eschrich 1988a).

Could cytoskeletal elements – actin filaments, microtubuli – actively perform *fast* shrinking, as suggested already by Toriyama and Jaffe (1972)? Although both types of proteins were localized to the *Mimosa* primary pulvinus (using antibodies against muscular actin and a protozoan tubulin; Fleurat Lessard et al. 1993), a combination of pharmacological and immunocytochemical approaches implicated only actin in the seismonastic responses, additionally indicating the involvement of its phosphorylation by a tyrosine kinase (distinct from a serine/threonine kinase; Kanzawa et al. 2006). Interestingly, the actin-depolymerizing agent cytochalasin D promoted stomatal opening by light and potentiated (independently of the activity of the H^+-ATPase) the activation (by hyperpolarization) of K^+-influx channels, and the filamentous-actin-stabilizing agent phalloidin inhibited stomatal opening and the activation of K^+-influx channels (Hwang et al. 1997), suggesting that actin may perhaps be involved not only in the "dramatic" movements of the pulvinus but also in the regulation of its "mundane", rhythmic (nastic) movements.

1.2.3 Plasma Membrane Transporters

What transporters are involved in the ion fluxes across the pulvinar cell membrane? Although it is obvious that the fluxes of K^+, Cl^- and water occur between the vacuole and the apoplast, i.e. across two membranes, there is practically no information about the tonoplast transporters of the pulvinar motor cells. So far, the function of only a few plasma membrane transporters in the pulvini has been observed in situ and partially characterized. Some of the details are given below.

1.2.3.1 H^+-Pump Activity

The activity of the proton pump in the plasma membrane in the *Samanea* pulvini was assayed indirectly via changes in the light-stimulated acidification of the medium bathing extensor and flexor tissues (Iglesias and Satter 1983; Lee and Satter 1989). Blue light (BL) acidified the extensor apoplast, consistent with pump activation, and alkalinized the flexor

apoplast, consistent with cessation of pump activity (Lee and Satter 1989). In accord with this, in patch-clamp experiments with intact *Samanea* flexor protoplasts, BL depolarized the flexor cells, probably by halting the action of the H^+ pump (Suh et al. 2000; but see the inexplicable opposite response in Kim et al. 1992). Red light or dark, following BL, activated the H^+ pump in flexors (acidifying the flexor apoplast and hyperpolarizing the flexor protoplast; Lee and Satter 1989, Suh et al. 2000), and inactivated the pump in extensors (alkalinizing the extensor apoplast; Lee and Satter 1989).

The motor cells of the *Phaseolus* laminar pulvinus (both extensors and flexors) reacted to BL in a manner similar to that of the *Samanea* flexors: shrinking (Koller et al. 1996), depolarizing (Nishizaki 1990, 1994) and alkalinizing their external milieu (as a suspension of protoplasts; Okazaki 2002). Vanadate, which blocks P-type proton ATPases, inhibited the BL-induced depolarization (Nishizaki 1994). Additionally, the inhibitory effect of BL was demonstrated directly on the vanadate-sensitive H^+-ATPase activity of membranes from disrupted *Phaseolus* pulvinar protoplasts (Okazaki 2002).

Extensors protoplasts isolated from the *Phaseolus coccineus* pulvinus reacted to *white* light (WL) and dark (D) similarly to extensors of *Samanea*: they swelled in WL and shrunk in D (Mayer et al. 1997). This, too, may be taken as indirect evidence of the activation/deactivation of the proton pump by WL and D respectively.

1.2.3.2 H^+/Cl^- Symporter

The presence of an H^+/anion symporter has been suggested based on experiments in which the net H^+ efflux from excised *Samanea* flexor tissue pieces, bathed in a weakly buffered medium, was greater with the impermeant iminodiacetate anions than with the permeant Cl^- in the external solution (Satter et al. 1987).

1.2.3.3 K^+-Release Channels

These channels are presumed to mediate K^+ efflux from pulvinar motor cells during their shrinking. Patch-clamp studies revealed depolarization-dependent, K^+-release (K_D) channels in the plasma membrane of pulvinar cell protoplasts (Moran et al. 1988; Stoeckel and Takeda 1993; Jaensch and Findlay 1998).

Ion Selectivity The selectivity for K^+ of the *Samanea* K_D channel was somewhat higher than for Rb^+, and much higher than for Na^+ and Li^+, and the channel was blocked by Cs^+, Ba^{2+}, Cd^{2+} and Gd^{3+} (Moran et al. 1990), and also by TEA (Moran et al. 1988). K_D channels in extensors were slightly less K^+ selective than in flexors (Moshelion and Moran 2000). Extensors and flexors

differed also in the details of the cytosolic Ca^{2+} sensitivity of the K_D channels gating, but the *overall* effect of cytosolic Ca^{2+} on these channels was rather minor (Moshelion and Moran 2000). By contrast, the *Mimosa* K_D channel currents, although generally similar in their voltage dependence and similarly blockable by external Ba^{2+} and TEA (Stoeckel and Takeda 1993), were severely attenuated (they "ran down") by treatments presumed to increase cytosolic Ca^{2+} (Stoeckel and Takeda 1995). Surprisingly, they were not blocked by external La^{3+} and Gd^{3+} at concentrations comparable to the blocking Gd^{3+} concentration in *Samanea*. In fact, both lanthanide ions *prevented* the "rundown" of the *Mimosa* K_D channels.

Regulation by Light Using patch-clamp, Suh et al. (2000) demonstrated an *increase* in the activity of K_D channels in cell-attached membrane patches of intact S*amanea* flexor protoplasts within a few minutes illumination with blue light, and a *decrease* in their activity within a few minutes of darkness, preceded by a brief red-light pulse (Fig. 1.3; Suh et al. 2000). No circadian control, however, was evident in the responsiveness of the flexor K_D channels to blue light. The authors resolved the blue-light effect in terms of two processes: (1) membrane depolarization-dependent K_D channel activation (a consequence of a blue light-induced arrest of the proton pump), and (2) a voltage-*in*dependent increase of K_D channel availability.

Molecular Identity Among the four putative K channel genes cloned from the *Samanea saman* pulvinar cDNA library, which possess the universal K channel-specific pore signature, TXXTXGYG, the *Samanea*-predicted protein sequence of SPORK1 is similar to SKOR and GORK, the only *Arabidopsis* outward-rectifying *Shaker*-like K channels. *SPORK1* was expressed in all parts of the pulvinus and in the leaf blades (mainly mesophyll; Fig. 1.1), as demonstrated in Northern blots of total mRNA. *SPORK1* expression was regulated diurnally and also in a circadian manner in extensor and flexor but not in the vascular bundle (rachis) nor in the leaflet blades (Moshelion et al. 2002a). Although the functional expression of SPORK1 has yet to be achieved, these findings strongly indicate that SPORK1 is the molecular entity underlying the pulvinar K_D channels.

1.2.3.4 K^+-Influx Channels

Using patch-clamp in the whole-cell configuration, Yu et al. described hyperpolarization gated K^+-influx (K_H) channels in the plasma membrane of *Samanea* extensor and flexor protoplasts (Yu et al. 2001). Paradoxically, these channels were blocked by external protons, contrary to what would be expected of channels presumed to mediate the K^+ fluxes during cell swelling which is concurrent with external acidification. This was particularly surprising in view of the external-acidification-*promoted* K^+-influx channels in

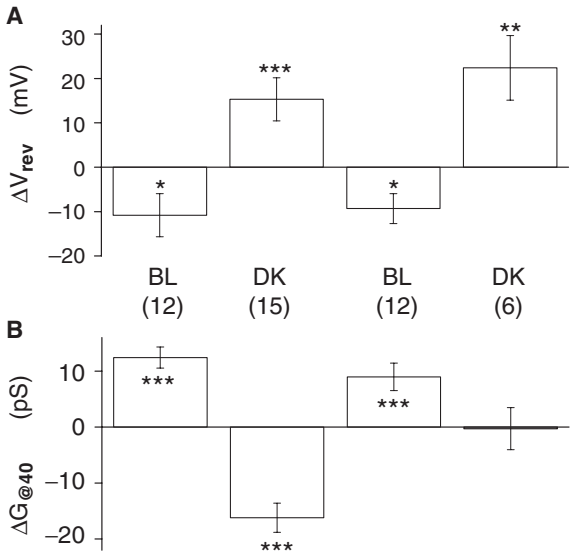

Fig. 1.3 Blue light enhances the activity of the *Samanea* K_D (K^+-release) channels in flexor protoplasts. **A** Light-induced shift of the membrane potential, manifested as shifts of the reversal potential, V_{rev} of K_D-channel currents in single cell-attached membrane patches during alternation between blue light (*BL*) and dark (*DK*). A negative shift of V_{rev} indicates membrane depolarization (mean±SE). The asterisks indicate the significance level of difference from zero: * $P<0.05$, ** $P<0.01$, *** $P<0.005$; *n* number of membrane patches. **B** BL-induced, membrane-potential-*in*dependent changes of K_D-channel activity, manifested as changes in G@40, the mean patch conductance at 40 mV depolarization relative to the V_{rev} of the patch (mean±SE). The asterisks and *n*, as in **a** (reproduced with permission, Suh et al. 2000)

guard cells (Blatt 1992; Ilan et al. 1996). The authors were able to resolve this paradox by quantitative comparisons of the actual vs. the required K^+ influx, in particular when they "recruited" into their calculations also the relatively large voltage-*in*dependent and acidification-insensitive leak-like currents recorded along with currents activated by hyperpolarization (Yu et al. 2001). No diurnal variation in the activity of the K^+-influx channel was noted in the patch-clamp experiments.

K^+-selective channels were reportedly observed during membrane hyperpolarization also in extensor protoplasts from pulvini of *Phaseolus* (Jaensch and Findlay 1998). However, hyperpolarizing pulses failed to activate such channels in protoplasts from the primary pulvini of *Mimosa* (Stoeckel and Takeda 1993).

Regulation by Light Kim et al. (1992) monitored membrane potential in isolated *Samanea* extensor and flexor protoplasts using the fluorescent dye DiS-C3(5) and pulses of elevated external K^+ concentration to specifically

detect states of high potassium permeability of the cell membrane (manifested as depolarization). They interpreted this high permeability as a high level of activity of K^+-influx channels (K_H channels). They were thus able to demonstrate an almost full (21 h-long) cycle of K^+-influx channel activity (in continuous darkness), which was out of phase in extensors and flexors, paralleling the periods of expected swelling in these protoplasts: the activity of the channels was high in extensors anticipating a "light-on" signal and during early morning hours, and in flexors anticipating "light-off" and in the evening (Kim et al. 1993). In addition, these authors demonstrated *circadian-enabled* (*gated*) responsiveness of extensors and flexors to light stimuli: during the second half of the night of a normal day cycle, blue light opened K^+-influx channels in extensors and closed them in flexors, and red light had no effect at all at this time. Then, during the last third of the day (of a normal day cycle), blue light opened these channels in extensors but had no effect on flexors, and darkness closed these channels in extensors (without red light) and opened them in flexors (when preceded by red light; ibid.).

Molecular Identity Two of the *Shaker* K-channel-like genes cloned from the *Samanea* cDNA pulvinar library are *SPICK1* and *SPICK2*, and their predicted protein sequences are homologous to AKT2, a weakly inward-rectifying *Shaker*-like *Arabidopsis* K channel. *KAT1* (or *KAT2*), genes of the chief K^+-influx channels of the *Arabidopsis* guard cells, were not detected in the pulvinar cDNA library in several repeated trials. Based on Northern blot analysis, the SPICK1 and SPICK2 transcript level is regulated diurnally (SPICK2 in extensor and flexor, SPICK1 in extensor and rachis), and their expression in the extensor and flexor is also under a circadian control (Moshelion et al. 2002b). Because circadian rhythm governs also the resting membrane K^+ permeability in extensor and flexor protoplasts and the susceptibility of this permeability to light stimulation (Kim et al. 1993), SPICK1 and SPICK2 are very likely the molecular entities underlying the activity of the in-situ K_H channels. *Samanea* pulvinar motor cells are thus the first described system combining light and circadian regulation of K channels at the level of transcript and membrane transport.

1.2.3.5 Ca^{2+} Channels

A K_D channel rundown (gradual loss of activity) was used as an indicator – as indirect evidence – for the influx of Ca^{2+}, and thus for the existence and function of hyperpolarization-activated Ca channels in the plasma membrane of protoplasts from pulvini of *Mimosa* (Stoeckel and Takeda 1995). Surprisingly, Gd^{3+} *prevented* this rundown (Stoeckel and Takeda 1995), rather than inhibiting the K^+-release channel by itself, as it did in *Samanea* (Moran et al. 1990).

1.2.3.6 Anion Channels

There is practically no information about anion channels in the pulvinar plasma membrane. Pharmacological evidence that Cl channels mediate ABA-induced shrinking of protoplasts isolated from a laminar pulvinus of *Phaseolus vulgaris* (Iino et al. 2001) is not conclusive, as NPPB (an inhibitor used in the study) has been shown also to inhibit plant K^+-release channels with an even higher affinity (Garrill et al. 1996).

1.2.3.7 Mechanical-Stretch-Activated Channels

Stretch-activated channels (SACs) have been detected by patch-clamp in cell membranes in virtually all cell types assayed, including procaryotes (see, for example, references in the review by Kung 2005). In *Samanea* flexors and extensors, these channels were observed quite frequently upon application of pressure to the patch-pipette, during and after the formation of a giga-seal between the patch-pipette and the protoplast membrane. Channels of undefined selectivity (cation-non-selective or anion-selective, but not specifically K^+-selective) were activated reversibly in outside-out patches by outwardly directed (i.e. membrane-extending) pressure pulses under 30 mm Hg. These stimuli were well within the physiological range of estimated turgor values occurring in the *Samaea* pulvini (Moran et al. 1996). The possible physiological role of these channels might be in volume regulation of motor cells, thus constituting a part of the rhythm-regulating process.

1.2.3.8 Water Channels (Aquaporins)

Water Permeability Water permeability (P_f) of the plasma membrane was determined in motor cell protoplasts of *Samanea* by monitoring their swelling upon exposure to a hypotonic solution. The P_f of the protoplasts was regulated diurnally, being the highest in the morning (extensor and flexor) and the evening (extensor), corresponding to the periods of most pronounced volume changes, i.e. the periods of highest water fluxes. P_f increases were inhibited down to the lowest, noon level by 50 μM $HgCl_2$ and by 250 μM phloretin, both non-specific transport inhibitors shown to inhibit aquaporins in some systems (Dordas et al. 2000), and by 2 mM cycloheximide, an inhibitor of protein synthesis. The susceptibility of P_f to fast modification by pharmacological agents has been interpreted as evidence for the function of plasma membrane aquaporins (Moshelion et al. 2002a).

Molecular Identity Two plasma membrane intrinsic protein homologue genes, *SsAQP1* and *SsAQP2*, representing two separate subfamilies of aquaporins, PIP1 and PIP2, were cloned from the *Samanea* pulvinar cDNA library

and characterized as aquaporins in *Xenopus laevis* oocytes. P_f was 10 times higher in *SsAQP2*-expressing oocytes than in *SsAQP1*-expressing oocytes, and *SsAQP1* was found to be glycerol permeable. In the oocytes, *SsAQP2* was inhibited by 0.5 mM $HgCl_2$ and by 1 mM phloretin. In the leaf, the aquaporin mRNA levels differed in their spatial distribution, with the most prominent expression of *SsAQP2* found in pulvini. The transcript levels of both were regulated diurnally in phase with leaflet movements. Additionally, SsAQP2 transcription was under circadian control. These results linked SsAQP2 to the physiological function of rhythmic cell volume changes (Moshelion et al. 2002a).

Two plasma membrane aquaporins PIP1;1 and PIP2;1, representing PIP1 and PIP2, as in *Samanea*, were isolated from a *Mimosa pudica* (Mp) cDNA library and characterized in heterologous expression systems, the frog oocytes and mammalian Cos cells. MpPIP1;1 alone exhibited no water channel activity but it facilitated the water channel activity of MpPIP2;1, and immunoprecipitation analysis revealed that MpPIP1;1 binds directly to MpPIP2;1 (Temmei et al. 2005). However, the relation of the *Mimosa* MpPIP1 and MpPIP2 to the rhythmic movement of the pulvinus (localization and function in the pulvinus) has yet to be demonstrated.

1.2.4 Tonoplast Transporters

The solutes and water traversing the plasma membrane cross also the tonoplast. Vacuoles appear to fragment and coalesce during leaf movements (Setty and Jaffe 1972). However, only one study addressed explicitly vacuolar transporters in pulvini.

1.2.4.1 H^+-ATPase

The only evidence so far for a proton transporter across a pulvinar tonoplast comes from immunolocalization studies in the primary pulvinus of *Mimosa* (Fleurat-Lessard et al. 1997). A catalytic α-subunit of an H^+-ATPase was detected abundantly and almost exclusively in the tonoplast of the aqueous (colloidal) vacuoles. The maturation of the pulvinus, and the acquisition of the very rapid responsiveness to external stimuli were accompanied by a more than threefold increase in H^+-ATPase abundance per length unit of membrane (Fleurat-Lessard et al. 1997).

1.2.4.2 An SV Channel?

SPOCK1, a homologue of the *Arabidopsis KCO1* (two-pore-in-tandem K-signature channel) cloned from the *Samanea* cDNA pulvinar library

(Moshelion et al. 2002b), may represent, similarly to KCO1, the cation-permeable, voltage-dependent SV channel of the tonoplast (Czempinski et al. 2002), or a K^+-selective voltage-*in*dependent vacuolar channel (under the new name of TPK1; Bihler et al. 2005). Yet, until firmly localized to the tonoplast, *KCO1* and *SPOCK1* should be also considered as a candidate plasma membrane channels (as in the case of the pollen TPK4 channel of the same family; Becker et al. 2004). *SPOCK1* mRNA level in the *Samanea* pulvini fluctuated under diurnal control (with the highest level in the morning) but not in constant darkness, and only in extensor and flexor (not in the rachis nor the leaflet blades; Moshelion et al. 2002b). Clearly, SPOCK1 function, localization and role in leaf movement await resolution.

1.2.4.3 Aquaporins

γ-TIP (tonoplast intrinsic protein) was detected in the membrane of aqueous (colloidal) vacuoles of *Mimosa* primary pulvinus using immunocytochemical approaches. Development of the pulvinus into a motor organ was accompanied by a more than threefold increase in aquaporin abundance (per length unit of membrane measured in electron microscopy micrographs), paralleling the development of the ability to respond rapidly to an external stimulus (Fleurat-Lessard et al. 1997). A single TIP aquaporin gene, *TIP1;1*, was cloned from *Mimosa* cDNA library, and its product, expressed in frog oocytes, conducted water (Temmei et al. 2005). Its identity with the γ-TIP of the pulvinus and its involvement in the pulvinar function have yet to be determined.

1.3 Mechanisms of Regulation

Membrane transporters are the end point in the signalling cascades regulating pulvinus movement. This regulation is rather complex (Fig. 1.4) and includes a large number of factors, such as light, the circadian clock, hormones and temperature. Such regulation occurs at both transcriptional and posttranslational levels (Fig. 1.4).

1.3.1 Regulation by Protein Modification – Phosphorylation

As yet, evidence for *rhythmic* phosphorylation of pulvinar proteins in situ is lacking. The accumulating information pertains to in-vitro assays or, at best, to acute stimuli. Nonetheless, this may be also one of the ways the clock affects transporters by, for example, *gating* their responsiveness to acute stimuli (see Sect. 1.2.3.4 above).

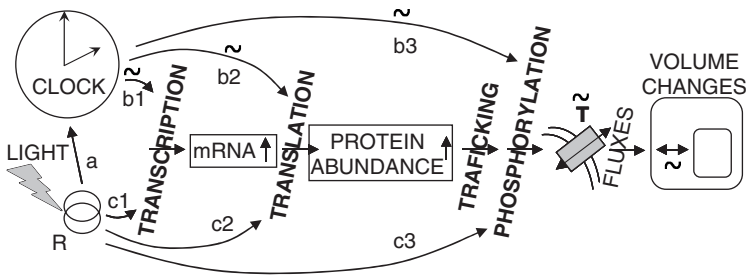

Fig. 1.4 Regulation of membrane transporters in the pulvinus at the levels of transcription, translation and protein modification (a schematic model). b*n* are clock output signalling pathways and a and c*n* are signalling pathways from the light-activated receptor, R. T is the transporter protein in the membrane. The processes affected are indicated. The other signs are as in Fig. 1.2

1.3.1.1 Phosphorylation of the Proton Pump

The recently discovered, immunologically undistinguishable three isophoto-tropins of the *Phaseolus vulgaris* pulvinus (see Sect. 1.3.2.2 below, and Inoue et al. 2005) were identified as the first element in the phototransduction cascade of shrinking pulvinar motor cells (Fig. 1.5). In the dark, they existed in a dephosphorylated state and the plasma membrane H^+-ATPase existed in a phosphorylated state. A 30-s pulse of blue light (BL) induced the phosphorylation of the phototropins and the dephosphorylation of the H^+-ATPase. Three findings indicated that these phototropins may function upstream of the H^+-ATPase and decrease the activity of H^+-ATPase by dephosphorylation: the phototropin phosphorylation peaked the earliest (Fig. 1.5a); the phosphorylation and dephosphorylation exhibited similar fluence rate dependencies on BL (Fig. 1.5b); inhibitors of the phototropin phosphorylation (the specific flavoprotein inhibitor diphenyleneiodonium and the protein kinase inhibitors K-252a and staurosporine) inhibited not only the phototropin phosphorylation but also H^+-ATPase *de*phosphorylation (Fig. 1.5c–f). This indicated that H^+-ATPase *de*phosphorylation depended on phototropin phosphorylation (Inoue et al. 2005).

Very interestingly, the *de*phosphorylation of the H^+-ATPase upon BL stimulation in the *Phaseolus* pulvinus was precisely the reverse of that occurring in the guard cell, where BL *stimulated* H^+-ATPase phosphorylation (Kinoshita and Shimazaki 1999) and activated the H^+-ATPase. Such contrast was manifested also in the reversed reactions of H^+-ATPase activity to BL illumination in flexors and extensors of *Samanea* (Lee and Satter 1989) – a decrease of H^+ secretion in *Samanea* flexor (albeit after a transient increase in activity; Okazaki et al. 1995) and activation of H^+ secretion in *Samanea* extensors (as in guard cells; Shimazaki et al. 1985).

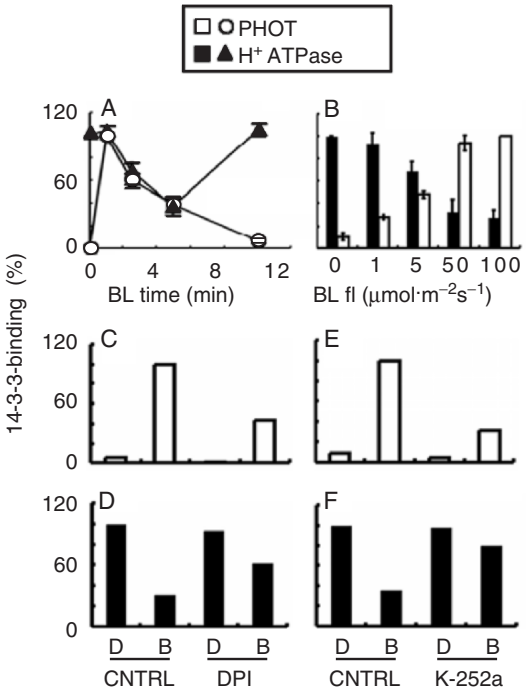

Fig. 1.5 Pulvinar phototropins mediate the dephosphorylation of the plasmalemmal H^+-ATPase by blue light. **A** Time courses of recombinant 14-3-3 protein binding to phototropin and to H^+-ATPase (as a measure of their phosphorylation status) in pulvinar microsomal membranes in response to a blue-light pulse (30 s at 100 fmol m^{-2} s^{-1}; mean±SE, *n*=3). **B** Blue-light fluence rate dependencies in the binding of 14-3-3 protein to the H^+-ATPase and to phototropin (a representative of three similar experiments). **C, D** The effect (relative to control, CNTRL) of 1 h pre-incubation (in the dark) of excised pulvini in flavoprotein inhibitor, DPI (100 μM) on the response to a blue-light pulse, B, compared to dark, D. **E, F** The effect of similar pretreatment with Ser/Thr protein kinase inhibitors, K-252a (10 μM) (reproduced with permission, Inoue et al. 2005)

1.3.1.2 Phosphorylation of Samanea K Channels

In-Situ Phosphorylation of the K_D Channel The enhancement of the activity of K_D channels in flexor protoplasts by blue light implicates a voltage-independent component, which could be a phosphorylation (see Sect. 1.2.3.3 above and Suh et al. 2000). Indeed, the activity of K_D channels in *Samanea* extensor protoplasts, assayed using patch-clamp in a whole-cell configuration and in inside-out patches (Moran 1996), required the presence of Mg^{2+} and ATP (or its kinase-hydrolysable analogue, ATP-γ-S) at the cytoplasmic surface of the plasma membrane. In their absence, channel activity decayed completely within 15 min, but could be restored by adding ATP and Mg^{2+}. A non-hydrolysable ATP analogue, AMP-PNP (5′-adenylylimidodiphosphate), did not substitute for ATP. H7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine), a broad-range

kinase inhibitor, reversibly blocked the activity of K_D channels in the presence of MgATP (ibid.).

In another series of experiments, several proteins in isolated plasma-membrane-enriched vesicles of *Samanea* extensors *and* flexors underwent phosphorylation without an added kinase in solutions similar to patch-clamp. The pattern of phosphorylation in the two cell types was not identical (Yu et al. 2006). These results strongly suggest that the activation of the outward-rectifying K channels by depolarization depends critically on phosphorylation by a kinase tightly associated with the membrane. However, it still remains unclear whether the K_D channel itself needs to be phosphorylated to function, or an accessory protein or even a lipid need to be phosphorylated. Support for the latter notion comes from a recent study in which the addition of $PtdInsP_2$ (phosphatidylinositol(4,5)bisphosphate) replaced MgATP in restoring the "run-down" activity of SKOR channels (the presumed *Arabidopsis* molecular equivalent of the *Samanea* K_D channels), in inside-out patches of a frog oocyte (Liu et al. 2005).

In-Situ Phosphorylation of the K_H Channel The voltage-dependent K^+-selective fraction of the inward current in extensor and flexor cell protoplasts (i.e. the activity of their K_H channels) has been investigated in whole-cell patch-clamp assays (see Sect. 1.2.3.4 above). The promotion of phosphorylation was achieved using okadaic acid, OA, an inhibitor of protein phosphatase types 1 and 2A. High levels of phosphorylation (300 nM of OA) inhibited K_H-channel activity whereas low levels of phosphorylation (5 nM of OA) promoted channel activity in flexors but had no effect in extensors (Yu et al. 2006). This difference between flexor and extensor in the susceptibility of their K_H-channel activity to phosphorylation may be related to their time-shifted contribution to the pulvinar movement.

In-Vitro Phosphorylation of SPICK2 The putative SPICK2-channel protein, the molecular candidate for the K_H channel (see Sect. 1.2.3.4), raised in cultured insect cells (Sf9), has been phosphorylated in vitro by the catalytic subunit of the broad-range cyclic-AMP (cAMP)-dependent protein kinase (PKA; Yu et al. 2006). Although this finding does not necessarily imply that PKA regulation of K_H channels is physiologically relevant, it is consistent with the notion that the SPICK2 channel (assuming it is a pulvinar K^+-influx channel) may be regulated in vivo by *direct* phosphorylation.

1.3.1.3 Phosphorylation of Water Channels

The water permeability of frog oocytes expressing solely MpPIP2;1, one of the two *Mimosa* plasma membrane aquaporins (see Sect. 1.2.3.8), was *in*dependent of phosphorylation. Its interaction (demonstrated by immunoprecipitation) with the water-*im*permeable MpPIP1;1 was also phosphorylation *in*dependent.

Yet, the water permeability of this complex increased in parallel to its phosphorylation – curiously, localized to Ser-131 of MpPIP1;1 (Temmei et al. 2005).

1.3.2 The Perception of Light

Plant photoreception has been reviewed recently by Wang (2005). Our focus here is on photoreception related to leaf movement. Where in the plant are the different light stimuli perceived? Are the acute and clock signals (Fig. 1.2) perceived via different receptors? Which are they? Physiological experiments delineated broad classes of receptors, and biochemical-molecular tools are only beginning to be applied in this area of research.

1.3.2.1 Phytochrome

Phytochrome-Mediated Responses A hallmark for a phytochrome-perceived red-light (and sometimes, blue-light) signal is its reversal by far-red light. Phytochrome mediates the phase-shifting of leaf-movement rhythms in various plants, e.g. in *Samanea* and *Albizzia* (Simon et al. 1976; Satter et al. 1981). It is also a receptor for acute signals: in *Samanea*, when red light preceded darkness, it enhanced leaf closure, transmitting a swelling signal to the pulvinar flexor cells (reviewed by Satter and Galston 1981). This signalling was replicated in isolated flexor protoplast (Kim et al. 1992, 1993). Moreover, phytochrome-perceived red light, followed by darkness, was thought to signal shrinking to pulvinar extensors (Satter and Galston 1981) but, in isolated extensor protoplast, red illumination (i.e., the resulting Pfr form of the phytochrome, see below) appeared to be unnecessary (Kim et al. 1992, 1993).

In the pulvinar protoplasts of *Phaseolus vulgaris*, the Pfr form of the phytochrome had to be present for the shrinking response to be induced by the blue light. Far-red light abolished the blue-light responsiveness, red light (preceding the blue) restored it (Wang et al. 2001).

In *Samanea*, in whole darkened pulvinar flexors illuminated with red light, phytochrome mediated hyperpolarization (measured directly) and, subsequently – upon illumination with far-red light – depolarization (Racusen and Satter 1975; see also Sect. 1.2.2.2).

Molecular Identity Phytochrome is a multi-gene protein (in *Arabidopsis*, it is denoted PHYA through PHYE) with a linear-tetrapyrrol cofactor, changing its conformation between a red light-absorbing form (Pr) and a far-red light-absorbing form (Pfr). A putative *Robinia* phytochrome A (PHYA) has been detected by immunoblotting pulvinar sections using an antibody to mustard (*Sinapis alba* L.) PHYA (CP 2/9). By contrast, an antibody against the cucumber (*Cucumis sativus* L.) phytochrome B (PHYB) (mAT1) did not produce any signal in these blots (Moysset et al. 2001). Thus, immunochemistry

suggests it could be PHYA-like. In further support of this notion, in tobacco (*Nicotiana plumbaginifolia*), the absence of PHYB in the *hlg* mutant did *not* prevent the normal entraining of the endogenous rhythm of growth movements of rosette leaves (although it did affect the sensitivity of bolting to photoperiod, i.e. to short-vs. long-day regimes; Hudson and Smith 1998).

On the other hand, a suggestion that the pulvinar phytochrome could be related to PHYB is based on an *Arabidopsis* nonsense *oop1* (out of phase 1) mutation in the PHYB apoprotein. This mutation caused defective photoreception and defective circadian phase setting in light–dark cycles (although it did not prevent normal entrainment by temperature cycles; Salome et al. 2002). A physiological hint in support of the latter notion is the low-fluence irradiance, in the range of 1 to 1,000 fmol m^{-2} s^{-1} of light, characterized by red/far-red reversibility (Wang 2005), effective in stimulating the known phytochrome responses of pulvinar cells (as, for example, in Moysset and Simon 1989; Kim et al. 1993).

Localization The phytochrome was localized to the pulvinar cells by examining pulvinar responses during selective illumination of different leaf parts and, even more convincingly, by demonstrating red/far-red responsiveness in isolated protoplasts (e.g. in *Samanea*, by Kim et al. 1992, 1993). Immunological evidence for a motor cell-specific localization was provided in *Robinia*. The labelling with anti-PHYA antibody (see above) was restricted to cortical cells and there was no evidence of labelling either in the vascular system nor in the epidermis. The pattern of labelling was the same in both extensor and flexor cells, irrespective of whether phytochrome was in the Pfr or in the Pr form (Moysset et al. 2001).

1.3.2.2 Blue-Light Photoreceptor

Blue Light-Mediated Responses Blue light, perceived by an unknown photoreceptor, can also shift the rhythm of leaf movement, although this requires illumination of a few hours. Acting "acutely", it is a "shrinking signal" to flexor cells and a "swelling signal" to extensor cells, causing leaf unfolding in *Samanea* and *Albizzia* (Satter et al. 1981).

In contrast to *Samanea*, in *Phaseolus vulgaris* blue light caused motor cell shrinking in the laminar pulvinus *on the irradiated side*, wherever it occurred, *irrespective* of the stereotyped division of the pulvinus into extensor (abaxial) and flexor (adaxial), causing the phototropic bending of the pulvinus towards the light source. Such bending orients the leaves, maximizing their light-receptive area, and probably accounts for movements of solar tracking described in *Phaseolus* (e.g. Berg 1986; Fig. 1.1c). Consistent with this, in protoplasts isolated from the *Phaseolus* laminar pulvinus, blue light evoked shrinking, without distinction between the extensor and flexor cells, but it required the presence of the far-red light-absorbing form of phytochrome (i.e. red-light pre-illumination; Wang et al. 2001).

The action spectrum of the depolarization recorded in the *Phaseolus* laminar pulvinus (concomitant with the initiation of shrinking signalling) peaked at 460 nm, with lower peaks at 380 and 420 nm. Almost no sensitivity was observed at wavelengths shorter than 360 nm and longer than 520 nm (Nishizaki et al. 1997). This earlier study found no red and far-red light effects on the depolarization of the motor cell, thereby excluding phytochrome participation in this movement.

A similar action spectrum was found for both diaheliotropic and paraheliotropic movements of greenhouse-grown soybean (*Glycine max*) seedlings. The action spectrum of movements of the pulvini of unifoliolate leaves – recorded by means of interference filters – peaked between 410 and 440 nm and between 470 and 490 nm (Donahue and Berg 1990). Indeed, blue light was found necessary for these movements. Thus, spectroscopic studies suggest that the pulvinar blue-light receptor is similar to the receptor involved in the general phototropic responses (reviewed by Briggs and Christie 2002).

Molecular Identity Recently, three genes of phototropins, *PvPHOT1a*, *PvPHOT1b* and *PvPHOT2*, have been cloned from the bean pulvinus, and their protein products demonstrated to be the pulvinar blue-light receptor(s) for the acute responses (Inoue et al. 2005). Their *Arabidopsis* homologues, PHOT1 and PHOT2, have been localized to the plasma membrane (Harada et al. 2003), suggesting the bean phototropins may be localized similarly. The pulvinar phototropins seem to participate in what appears to be the first step of phototransduction, causing – through unknown step(s) – the dephosphorylation of the plasma membrane H^+-ATPase.

The intriguing question is – are the photoreceptors which feed into the clock the same as those mediating the acute responses? With respect to phytochrome, an affirmative answer appears to receive support from findings in *Arabidopsis*. Here, a physical interaction was demonstrated between the C terminal fragments of phytochrome B (PHYB) and the "clock oscillator proteins", Zeitlupe (ZTL) and cryptochrome 1 (CRY1; Jarillo et al. 2001).

Also phototropins may mediate blue-light signals to the clock. This is suggested by the finding that, in *Arabidopsis*, the double mutant lacking the cryptochromes cry1 and cry2, and even a quadruple mutant lacking the phytochromes phyA and phyB as well as cry1 and cry2, retained robust circadian rhythmicity, as reflected in the growth movements of the cotyledons. Moreover, this movement could still be phase-shifted by [unspecified, but apparently white] light; i.e. despite being nearly "blind" for developmental responses, the quadruple mutant perceived a light cue for entraining the circadian clock (Yanovsky et al. 2000).

1.3.3 Intermediate Steps

The best established second messenger in plant signalling is cytosolic Ca^{2+} (Hetherington and Brownlee 2004). It has been the central focus in most studies of signalling in the pulvinus, with attempts to confirm it as part of the

phosphatidylinositol (PtdIns) signalling pathway. The possible target effectors of Ca^{2+} may be calmodulin, actin and annexins; these have only begun to be examined in pulvini.

1.3.3.1 The Involvement of Calcium

Pharmacological Alteration of Rhythm Applying effectors of Ca^{2+} to pulvini interfered with their rhythms as well as with their acute responses to illumination. EGTA, a Ca^{2+} chelator, applied to *Phaseolus vulgaris* primary pulvinus, suppressed its circadian movements (strongly depending on the phase of application; Kayali et al. 1997). Various calmodulin (CAM) antagonists (chlorpromazine (CPZ), trifluoperazine (TFP), calmidazolium and N-(6-aminohexyl)- 5-chloro-1-naphthalenesulfonamide (W-7), but not W5, the inactive analogue of W7), and also 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate hypochloride (TMB-8, an inhibitor of intracellular IP_3-mediated cytosolic calcium mobilization; Schumaker and Sze 1987) – all shifted the circadian phase of the *Robinia pseudoaccacia* leaflet movement in continuous darkness, characterized by phase response curves (PRCs). The amplitudes of the advances were proportional to the concentrations of the agents. All these antagonists produced PRCs somewhat similar in shape to the PRC produced by 2-h pulses of blue light, but only TMB-8 produced a PRC almost identical to the blue-light PRC, with advances during the subjective day and delays during the subjective night (Gomez et al. 1999). Interestingly, applying agents presumed to *increase* the internal Ca^{2+} concentration, such as calcium ionophore A23187 and, separately, 2-h pulses of 10 mM $CaCl_2$, created PRCs almost identical to the PRC of 15 min of red light, with delays during the subjective day and advances during the subjective night, i.e. the reverse to that of blue light (Gomez and Simon 1995).

Pharmacological Alteration of Acute Responses Acute effects of red light and blue light were also altered by applying Ca^{2+} effectors to whole pulvini *of Albizzia* and *Cassia* (Moysset and Simon 1989; Roblin et al. 1989). Most instructive, however, was a pharmacological study conducted on isolated extensor protoplasts of *Phaseolus coccineus* during their swelling and shrinking in a regime of 9 h light/15 h dark (which paralleled their expected behaviour in the intact pulvinus; Mayer et al. 1997). *Light-induced swelling* required Ca^{2+} influx from the surrounding medium. Promoting Ca^{2+} influx from outside elicited swelling in the dark, mimicking the "light-on" signal. *Dark-induced* shrinking occurred in Ca^{2+}-free medium, but was sensitive to manipulations of Ca^{2+} release from internal stores via the activation or inhibition of the PtdIns pathway, suggesting that the shrinking signal "light-off" is – but the swelling signal "light-on" is not – transduced through PtdIns hydrolysis and Ca^{2+} release from internal stores. However, increasing internal Ca^{2+} in the light could not substitute for the "light-off" signal, although it

could nullify the inhibition (by TMB-8) of mobilization of cytosolic Ca^{2+} in the presence of the "light-off" signal. Thus, although Ca^{2+} itself is *necessary*, it is *not sufficient* for shrinking, and "light-off" provides this additional required element (Mayer et al. 1997).

Phytochrome Phytochrome has been shown directly to increase cytosolic Ca^{2+} in other systems. For example, in etiolated wheat leaf protoplast, red light evoked Ca^{2+} increase mediated by phytochrome, associated with protoplast swelling (Shacklock et al. 1992). However, no such evidence has been obtained for pulvinar cells.

Phototropins In protoplasts isolated from motor cells of *Mimosa pudica* pulvini, UV(A) light (360 nm; possibly perceived by phototropins) increased transiently the cytosolic free Ca^{2+} concentration. This Ca^{2+} increase was not significantly modified when protoplasts were incubated in a nominally calcium-free medium and was not inhibited by calcium influx blockers ($LaCl_3$ and nifedipine), arguing for a mobilization from intracellular stores (Moyen et al. 1995). The blue light-induced movement of the primary pulvinus of *Mimosa* is similar to the seismonastic response in terms of its direction and in the underlying loss of osmoticum (Stoeckel and Takeda 1993).

This distinct response to UV(A) resembles the PtdIns pathway-related response mediated by phot2 in de-etiolated *Arabidopsis* seedlings. Here, while both phot1 and phot2 could induce Ca^{2+} influx from the apoplast through a Ca^{2+} channel in the plasma membrane in response to blue light (phot1, at lower fluence rates: 0.1–50 mmol m^{-2} s^{-1}, and phot2, at higher fluence rates: 1–250 mmol m^{-2} s^{-1}), phot2 alone induced phospholipase C-mediated phosphoinositide signalling (Harada et al. 2003).

Circadian Ca^{2+} Oscillations Circadian Ca^{2+} oscillations would seem inevitable in the mature pulvinar cells, in view of the strong evidence for the involvement of Ca^{2+} in the rhythmic movements (see above). However, so far in plants, they have been documented only in tobacco (*N. plumbaginifolia* and in *Arabidopsis* seedlings; Fig. 1.6, and Johnson et al. 1995; see also the review by Hetherington and Brownlee 2004; Love et al. 2004), most likely in synchrony with the growth movements of the cotyledons. Circadian oscillations in free Ca^{2+} were not detected in nuclei (Wood et al. 2001), and thus it is not clear how the cytosolic oscillations communicate – as an input to and/or as an output from the clock, the core elements of which (LHY, CCA1 and TOC1) reside only in the nucleus (Dodd et al. 2005, and references therein).

1.3.3.2 PhosphatidylInositides (PIs)

Although the paradigm in animals should not be extended uncritically to plants, the general scheme for signal propagation via the PI pathway has

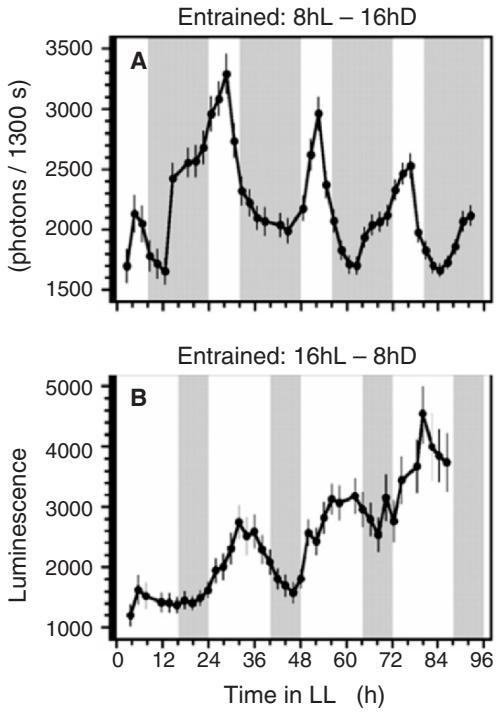

Fig. 1.6 Circadian oscillations of $[Ca^{2+}]_{cyt}$ in *Arabidopsis* seedlings entrained to different photoperiods. Aequorin luminescence emitted by seedlings kept under 110 fmol m^{-2} s^{-1} constant light (*LL*). Shown are measurements from seedlings entrained in 8L/16D (**a**) and 16L/8D (**b**) for 11 days before LL. During the entrainment light period, the photon flux density was 60 fmol m^{-2} s^{-1}. *Dots* represent the mean±SE bioluminescence of 12 seedling clusters. *Open areas* indicate the subjective day, and *shaded areas* the subjective night (reproduced with permission, Love et al. 2004)

received considerable support in plants (reviewed in Cote et al. 1996; Drobak et al. 1999; Stevenson et al. 2000; Hetherington and Brownlee 2004). Plants possess most of the enzymes producing the different phosphoinositides. Changes in free cytosolic Ca^{2+} concentration, when attributed to mobilization from internal stores, suggest the activation of the PtdIns pathway, in particular the hydrolysis of $PtdInsP_2$ by phospholipase C (PLCδ) into diacylglycerol (DAG) and IP_3. This has been confirmed in some cases by pharmacological agents, such as PLC inhibitors, and also in direct lipid assays (see Stevenson et al. 2000). Indeed, light, when it served as a cell-shrinking signal, increased the level of IP_3 in motor cells of leaf-moving organs (Morse et al. 1987; Kim et al. 1996; Mayer et al. 1997).

In addition to affecting the activity of PLCδ, light could affect other enzymes, too. For example, in etiolated sunflower hypocotyls, light transiently down-regulated the activity of PIP 5-kinase, consequently down-regulating

the level of its product, $PtdInsP_2$ (Memon and Boss 1990). Remarkably, in protoplasts isolated from cultured tobacco cells transformed with a human type I PIP 5-kinase, which had increased levels of PIP_2 and IP_3, the initial osmotic water permeability of the plasma membrane was twofold higher than that of protoplasts from control cells (wild type, or transformed with the plasmid without the kinase gene). The increased water permeability of the membranes appears to have been caused by the increased level of inositol phospholipids (Ma et al. 2005).

PIs in the Leaf-Moving Motor Cells In *Samanea*, 15 s of white-light illumination to the pulvini was sufficient to accelerate the turnover of phosphoinositides in the motor tissues (Morse et al. 1987). Furthermore, in *Samanea* pulvinar protoplasts, cell-shrinking stimuli applied at the appropriate circadian time (darkness, to the pulvinar extensors during the last third of the day period, or blue light, to the pulvinar flexors during the second part of the night) increased inositol 1,4,5-trisphosphate [Ins(1,4,5)P-3]. This "shrinking light" effect was *inhibited* by neomycin, at a concentration of 10 μM which inhibits $PtdInsP_2$ hydrolysis, and mimicked by mastoparan, a G-protein activator (Fig. 1.7, and Kim et al. 1996, and Moran et al. 1996). In parallel, the K^+-influx channels were shown to close in response to the same leaf-closing stimuli, i.e. in the protoplasts with increased Ins(1,4,5)P-3 levels (Kim et al. 1993, 1996). The authors concluded from these results that a phospholipase C-catalyzed hydrolysis of phosphoinositides, possibly activated by a G protein, was an early step in the signal-transduction pathway by which blue light and darkness closed K^+-influx channels in [the appropriate] *Samanea* pulvinar cells (Kim et al. 1996).

1.3.3.3 Annexins

Annexins are Ca^{2+}-, phospholipid- and protein-binding proteins, conserved evolutionarily between plants and animals, with an increasingly broad range of signalling functions revealed to date, including extracellular reception (Gerke and Moss 2002; Cantero et al. 2006), and nucleotide-induced oligo- (possibly tri-) merization of annexin6 to form active ion channels (of unspecified selectivity; Kirilenko et al. 2006). In plants, annexins have been predicted to form hyperpolarization-activated Ca channels (Hofmann et al. 2000; White et al. 2002) – this rather exotic function awaits further confirmation.

Annexins may also mediate Ca^{2+} effects. Eight annexin genes have been found in *Arabidopsis* (Cantero et al. 2006). Annexin protein isolated from *Mimosa* was found to bind in vitro to a phospholipid and to F-actin in the presence of calcium, its abundance being developmentally regulated. In the primary pulvinus during daytime, the amount of annexin increased with ABA concentration between 1 and 75 μM (but was not affected by cold nor mechanical stimuli!). Annexin abundance increased also at night, and its

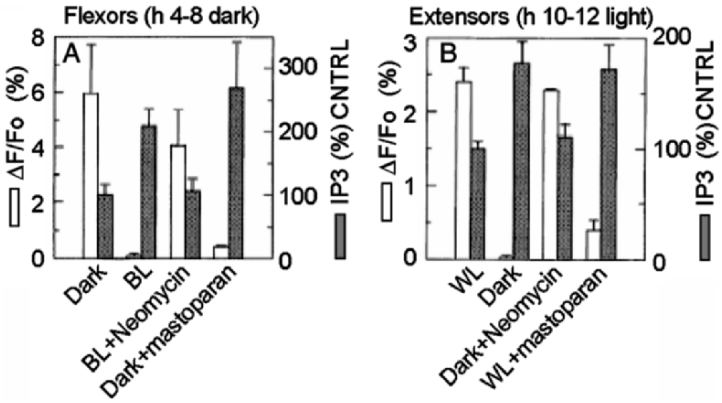

Fig. 1.7 Inositol,4,5 trisphosphate (IP_3) and K^+ permeability of protoplasts in response to shrinking signals. Protoplasts were isolated during the light period and transferred to the growth chamber at time of normal "light-off" (means±SD of three or four separate experiments, each done in duplicate). **A** Flexor responses to blue light (*BL*) at hours 4–8 of dark period. Cells were treated with indicated agent, and the change in fluorescence of the membrane-potential indicator dye 3,3′-dipropylthiodicarboxycyanide iodide was measured as a function of time after addition of 200 mM K^+. ΔF/Fo are values of fluorescence changes (relative to baseline) observed 30 s after addition of K^+. An increase in ΔF/Fo indicates that K^+ can enter the cell and depolarize membrane potential; K^+ channels are presumed to be open. No change in ΔF/Fo indicates that K^+ cannot enter the cell; channels are presumed to be closed. IP_3 levels were assayed in extracts prepared from similarly treated protoplasts. Values are presented as percent of untreated controls and represent IP_3 levels in protoplasts after 30-s treatment with blue light (10 μM neomycin) or 120-s treatment with 10 μM mastoparan. **B** Extensor responses to darkness at hours 10–12 of light period. Treatments were as above, except that darkness (rather than blue light) served as signal. *WL* White light (reproduced with permission, Moran et al. 1996)

distribution changed from the cell periphery during the daytime to cytoplasmic at night (Hoshino et al. 2004). It is thus interesting that, while actin (which binds to annexin) is thought to be involved in the seismonastic function of this pulvinus, annexin appears to be associated rather with nyctinastic transitions (but see also Sect. 1.2.2.3).

1.3.4 Regulation by Other Effectors

1.3.4.1 Hormones

Auxins (indole-3-acetic acid, IAA), gibberellins (GA_3) and ethylene have been found in gravistimulated leaf sheath pulvini of grasses (Brock 1993). These hormones, and also jasmonic acid and abscisic acid, affected the long-term growth responses of these tissues (in particular, cell elongation and cell wall production) following exogenous application (Montague 1995, 1997).

In the *non*-growing pulvini of legumes, only the acute effects of exogenous hormones on the leaf movements have been addressed (Bialczyk and Lechowski 1987; Bourbouloux et al. 1992; Mayer et al. 1997). IAA applied to whole pulvini opened *Cassia fasciculata* leaflets in darkness; pharmacological agents aimed to increase the cytoplasmic Ca^{2+} concentration promoted this opening whereas those agents aimed to decrease Ca^{2+} concentration, or to decrease its effect, were inhibited (although verapamil and nifedipine were ineffective; Bourbouloux et al. 1992).

IAA and ABA applied to protoplasts isolated from the laminar pulvinus of *Phaseolus vulgaris* and bathed in a medium containing KCl as the major salt affected both flexor and extensor cells similarly: protoplasts swelled in response to IAA and shrunk in response to ABA. Swelling depended on the presence of K^+ and Cl^- at acidic pH, and shrinking depended on the activity of a functional Cl channel (Iino et al. 2001), consistent with the accepted view of the "osmotic motor". No receptors for the hormone function are known in pulvini.

1.3.4.2 Turgorins

Turgorin, PLMF 1 (Periodic Leaf Movement Factor 1, sulfonated gallic acid glucoside) induces closure of leaflets in *Mimosa*, with a dose-dependent rate. PLMF 1 has been found in many higher plants with nyctinastic movements, including *Mimosa pudica*, and in a few plants with thigmonastic movements (as reviewed by Schildknecht and Meier-Augenstein 1990). Furthermore, since only one of two PLMF 1 enantiomers was active, a reaction with a specific receptor has been proposed (Kallas et al. 1990). The co-localization of the enzyme sulfonating the gallic glycoside, along with its end product, to the phloem cells in the motor organ suggested that this is the site of synthesis and/or accumulation of PLMF-1, supporting the hypothesis that PLMF-1 may be acting as a chemical signal during the seismonastic response of *Mimosa* (Varin et al. 1997).

1.3.4.3 Temperature

The circadian leaf rhythm is "temperature compensated", i.e. similarly to other clock manifestations, it has a constant period and phase over a certain range of temperatures. The underlying mechanism for this stability versus fluctuating temperature operates despite the strong dependence of circadian periods on the turnover of clock mRNA or clock protein (without compensation, rapid turnover of clock mRNA or clock protein would result in short periods, and slower turnover in longer periods; Ruoff et al. 1997).

Yet, similarly to entrainment by light, the clock may be entrained by temperature pulses, although their input pathways to the clock are different. For example, a mutation rendering the plant irresponsive to light preserved

its responsiveness to temperature entrainment (Salome et al. 2002). The receptor for temperature entrainment in plants is still an enigma – could it be an ion channel, or class of ion channels, similar to the heat- or cold-sensing TRP channels in mammals (Voets et al. 2004)?

1.4 Unanswered Questions

The light-signalling transduction steps converging on the "osmotic motor" have been outlined schematically in Fig. 1.8. To date, the information is scarce. In fact, most of the questions below have not yet been answered, or adequately answered, in any plant system.

The "osmotic motor" framework is not very mysterious but its functions still seek transporters: most of the transporters in the plasma membrane and in the tonoplast are yet to be defined and characterized physiologically, and identified molecularly.

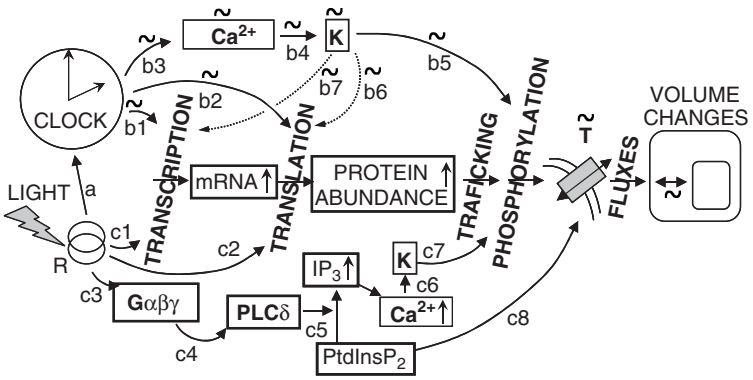

Fig. 1.8 Ca^{2+} involvement in the volume changes (a [partial] schematic model). b*n* are clock output signalling pathways and c*n* are the "acute" signalling pathways from the light-activated receptor, R. The acute "shrinking signalling" includes the PtdIns pathway, starting with the activation (c3) of a trimeric G protein (Gαβγ), followed by the activation (c4) of phospholipase C (PLCδ) and formation of second messengers by breakdown (c5) of $PtdIns_2$. Kinase (K) can be activated (c6) by the increased (↑) concentration of free cytosolic Ca^{2+} and phosphorylate (c7) transporters (T) or their modifying protein(s), including those involved in trafficking. $PtdInsP_2$ may affect some of the transporters directly (c8). Some of these elements and links have been demonstrated in the pulvinar cells. The clock may originate Ca^{2+} oscillations (b3), via the PtdInsP2 pathway or via a ryanodine-receptor/cADP-ribose pathway. Ca^{2+} effects (b4–b5) may resemble those of the acute pathway (c6–c7). The clock effects on the transporters may be "enabling", modulating (gating) its responsiveness to the acute signals. This enabling effect may be exerted also through other levels, marked by *dotted arrows*. See text for details and Fig. 1.4 for explanations of other signs

1.4.1 Acute, Fast Signalling

Signalling pathways in pulvini are practically unknown, and only the PtdIns pathway has begun to be unravelled. Therefore, there is a long list of questions to be answered.

Is ABA a *physiological* mediator of *shrinking* in pulvinar cells? Or is it serendipitously coupled to the same shrinking cascade as that of a "shrinking light" signal? What signals bring about the hypothesized increases of cytosolic Ca^{2+}? Is IP_3 or IP_6 the actual Ca^{2+}-mobilizing messenger? What is its receptor? Are cADP-ribose and the ryanodine receptor part of the Ca^{2+} mobilizing cascade in the pulvini? Does light signalling affect different enzymes of the PtdIns pathway? Do PtdIns lipids (e.g. $PtdInsP_2$) affect transporters directly? Could there be another pathway of ABA action (Levchenko et al. 2005)? Our [unpublished] evidence from another plant system suggests that $PtdInsP_2$ and/or IP_3 increase the activity of the plasma membrane aquaporins.

The following questions are only a partial list. The acute effects on transcription or translation, or membrane trafficking, unrelated to the clock and indicated in the model, all obviously invite additional questions.

The signalling cascade leading to *swelling* is also not known, apart from a requirement for calcium (Mayer et al. 1997) – and for a *timed* "enabling" function, such as the gated activity of the K^+-influx channels (Kim et al. 1993). Is it possible that via the lysis of phosphatidylcholine (PtdCh) by PLA_2, the resulting products – lysoPtdCh and free fatty acids – constitute the second messengers for motor cell swelling (Lee et al. 1996)? Exogenous PLA_2 caused premature swelling of *Samanea* flexor protoplasts whereas PLA_2 inactivated by a short pre-incubation with its inhibitor, manoalide, was inactive (Lee et al. 1996). As a plausible hypothesis, IAA may be a physiological mediator of the swelling light signals. Other hormones may be also involved. Since the phytohormone *brassinolide* appeared to increase the osmotic water permeability of aquaporins in *Arabidopsis* hypocotyl protoplasts (Morillon et al. 2001), and since aquaporins seem to be part of the "osmotic motor" of leaf movement, it might be of interest to examine whether brassinolide acts also in pulvini.

How is G protein involved in pulvinar signalling? What is the role of lipids such as sphingosine? The "guard cell paradigm" (e.g. Jones and Assmann 2004; Pei and Kuchitsu 2005; Coursol et al. 2005) is indeed an inspiring framework for studying the fast, acute pulvinar signal transduction.

1.4.2 The Clock Input and Output

There seems also no end to questions which may be addressed with regard to the regulation of pulvinar movements through the clock and by the clock. Studies of the interactions of the osmotic motor with the clock (roughly suggested in the model, Fig. 1.8) can perhaps be modelled based on growth

regulation via transcriptional regulation by light and the clock (such as suggested by Mizuno 2004, and by Nozue and Maloof 2006). An intriguing question still lingers, posed already several decades ago, and apparently nearly forgotten in the flurry of recent discoveries of clock molecular components – do membrane ion channels feed back into the circadian oscillator?

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2 The Pollen Tube Oscillator: Integrating Biophysics and Biochemistry into Cellular Growth and Morphogenesis

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Abstract

Individual cells constitute the minimal organization level to generate ultradian rhythms. A cell biology approach is thus necessary to better understand the intrinsic nature of these natural oscillators and their evolutionary significance. In this respect, pollen tubes provide a useful working model because, unlike other cells, their growth can be conveniently followed *in vitro* and it is known to involve both structural, biochemical as well as biophysical oscillators.

As in any other complex system, these oscillations involve almost all cellular components but, in this case, no causal role has yet been identified. Most studies consider growth as the reference for statistical correlation analysis with other oscillating parts, interpreted as an effect if correlated before growth and as a consequence when correlated after growth. Today, it is known that this group of oscillating variables include at least ion fluxes and internal free concentrations (calcium, chloride, protons and potassium), the cytoskeleton, membrane flow and wall synthesis. Despite the progress made in this domain, however, a central core-controlling mechanism is still missing, and even less is know about how all components interact to produce the macroscopic outcome, i.e. structural organised apical growth. In other words, we can see the arms of the clock and many underlying moving parts but still miss which work as pendulum, escapement and anchor.

Here, we review the recent advances in this field and critically address some of the pitfalls and inconsistencies in the data presently available. Some conceptual outlines and future directions of research are also discussed.

2.1 Finding Stability in Instability

For a long time, the view of a biological system as an equilibrium state-dependent structure has led researchers to use discrete time intervals and to statistically treat the average of the observations as a close reflection of reality.

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Rhythms in Plants: Phenomenology, Mechanisms, and Adaptive Significance

All deviations were regarded either as being random variations around "reality" in its progress towards a stable equilibrium point or as an unavoidable background noise which was to be disregarded.

This simplistic view is gradually changing, as increasing importance is being given to non-equilibrium dynamics in biological systems. Progress in this field can be attained only by the use of new methodology enabling in vivo monitorization on a nearly continuous timescale as well as different mathematical tools of analysis developed to this effect (Feijó et al. 2001).

The existence of specific non-linear behaviours, such as oscillations or pseudo-chaotic variations, has been verified in a wide range of biological processes. These include calcium waves in plant and animal cells, metabolite production oscillations in yeast, waves of cell aggregation in *Dictyostellum* or lamellipodial contractions in spreading and migrating animal cells as well as circadian clock-dependent behaviours (Goldbeter 1997).

The generalised view is that, at a biological level, these complex behaviours exist in order to attain homeostasis. Therefore, they would be characterized by tending to a stable equilibrium state, with the property that any deviation from that state would have a tendency to diminish in the *continuum* and to converge to the initial state. This is often called an "attractor", in the sense that there would be a set of conditions under which the regulatory capacity of a system would be maximal and, thus, would "attract" the system into a given dynamical status. Under these conditions, any perturbation would result in an approximation to the initial state after transient changes. This also introduces a certain degree of plasticity to the systems, which can be of advantage when dealing with unpredictable natural conditions. Most of these attractors have, in fact, some sort of rhythmicity, often as a defined oscillation. The maintenance of rhythmic behaviours, however, implies the existence of a tight control through a self-organizing mechanism, involving feed-forward and/or feedback regulatory loops.

To understand the viability of this putative "instability" in a living cell, one needs to consider all the inherent complexity of such a structure, both molecular and functional, where several complex pathways work simultaneously and in parallel, regulating (as well as being regulated by) numerous feedback loops. Due to this inner complexity, living cells appear as a perfect stage for oscillatory/chaotic behaviour to occur. At a theoretical level, it seems fairly simple to conceive this non-linear dynamics: essentially, one needs only two independent variables and a manner to regulate their interactions in the form of some non-linear term in an equation (Goldbeter 1991).

Oscillatory behaviours, such as the ones which form the focus of this chapter, are usually under the influence of "limit-cycle" attractors. In a phase-space plot, such as shown in Fig. 2.1, oscillations correspond to the evolution of the system towards a closed curve, the regulatory limit cycle, which is independent of the initial conditions. When at this dynamic condition,

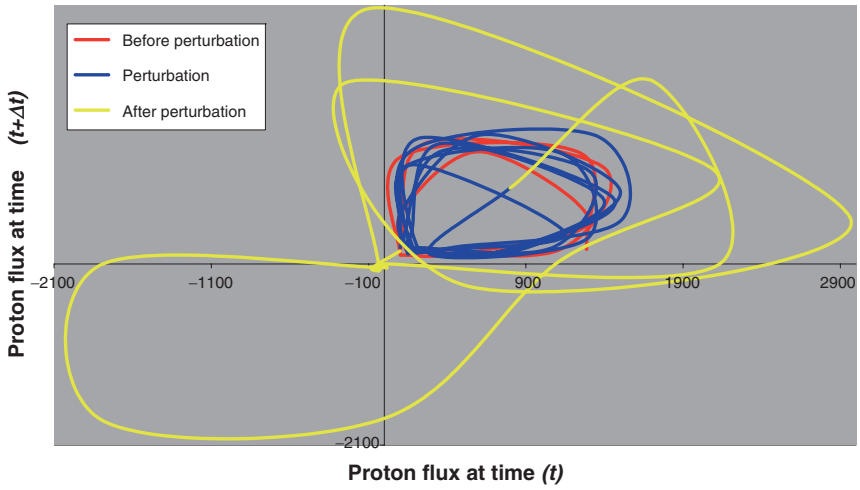

Fig. 2.1 Phase-space reconstruction using a time series of proton influx at the tip of a growing pollen tube. In a simplified definition, the "phase-space" representation is a graphic tool for analyzing the dynamics of a complex non-linear system. It is aimed at representing the dynamic relations between the independent variables of a system, expressed by trajectories towards a stable dynamic state. When the measured variables are not independent, each variable can be simply represented in a delay map. In a time series with a constant temporal shift (d*t*), every value is plotted against the next one in the series; the resulting plot denotes the evolution of the system. Note the aggregation of cycle trajectories in a restricted area of the phase-space, defining an attraction domain which, in this specific example, is a limit-cycle attractor. The convergence of trajectories in time represents a homeostatic behaviour of this system, and is by itself a graphic representation of its dynamic regulation characteristics. The homeostatic characteristic of the pollen tube limit-cycle attractor is well expressed in the experiment depicted, in which readings were acquired at 3-s intervals. Starting from its initial condition (*red line*), a perturbation was imposed by using a micropipette to double the concentration of calcium close to the tip. Immediately after the injection, the pollen tube underwent a series of undershoot/overshoot fluctuations (*yellow line* starting at the *black arrowhead*), likely to correspond to membrane de/hyperpolarization, transiently resulting in reverse, mixed or null fluxes. After three cycles, however, the system entered the limit-cycle area (*black arrow*) and became engaged in the original limit-cycle again (*blue line*; the *blue* and *red arrows* represent time-course direction; adapted from Feijó et al. 2001)

the key homeostatic parameters of the system should oscillate with a period equal to the time length of a full cycle. This period then becomes the most easily accessible variable usually used to study the regulatory properties of systems displaying such dynamical behaviour (Goldbeter et al. 1990).

Pollen tubes are one of the simplest biological structures in which oscillatory patterns have been reported, early becoming an exciting model for studies on the generators of periodicity, but dealing also with its importance at the level of cellular growth and morphogenesis.

2.2 Why Pollen Tubes?

The pollen tube is a unicellular structure which appears in the life cycle of higher plants to deliver the male gametes to the ovule, enabling fertilization and the completion of the plant sexual reproduction cycle (Boavida et al. 2005a, b). To reach the ovules, the pollen tube needs to elongate through the stigma and style, a distance sometimes several centimetres long, with a very fast growth rate and without cell division. Pollen tubes are polarised cells, and this polarisation underlies their most distinctive features. They grow exclusively at the apex (apical growth) by vesicle exocytosis and *de novo* cell wall synthesis. Internally, pollen tubes are characterized by an intense reverse-fountain type of cytoplasmic streaming, differential organelle distribution along the tube length, and size sorting of large organelles, which become excluded from the apex cytosol, forming the so-called clear cap (Fig. 2.2a; Boavida et al. 2005b).

Underlying this polarity, pollen tubes show a conspicuous polarisation of ion dynamics, expressed in the polarization/activation of specific channels/pumps, which results in well-defined membrane domains with specific ion-pumping characteristics (Fig. 2.2b; e.g. Feijó et al. 1999, 2001, 2004; Zonia et al. 2001, 2002). These are presumably involved in maintaining cellular homeostasis in specific places and may give rise to different ionic gradients along the pollen tube which seem to be necessary for growth (recently reviewed by Holdaway-Clarke and Hepler 2003). Somehow interrelated with these, membrane flow and the actin cytoskeleton are also highly dynamic (Fig. 2.2c). What makes pollen tubes an interesting and unique system is that all these components oscillate with the same period. Not surprisingly, their growth rate, which corresponds to the macroscopic outcome of all interacting parts, also oscillates distinctively with the same period, thus constituting an experimental time cue to which all other oscillations can be compared in terms of phase offset. This potentially allows us to test for causal relationships on a temporal sequence basis, aimed at developing a complex and fascinating paradigm of cell growth and morphogenesis.

Pollen tubes are also fairly easy to obtain, maintain and experimentally manipulate, and studies using these as model can be extended to other types of cells, from fungal hyphae to specialized animal cells with apical growth (Palanivelu and Preuss 2000). All these advantages largely explain why pollen tubes have increasingly attracted many research groups as models of choice (Feijó et al. 2004; Boavida et al. 2005a, b).

2.3 Growth Oscillations: Trembling with Anticipation?

In nature, few cells need to elongate as dramatically as a pollen tube on its journey to the female ovule. Since ovule fertilization proceeds on a first-come, first-served basis, and in order to compete successfully, individual

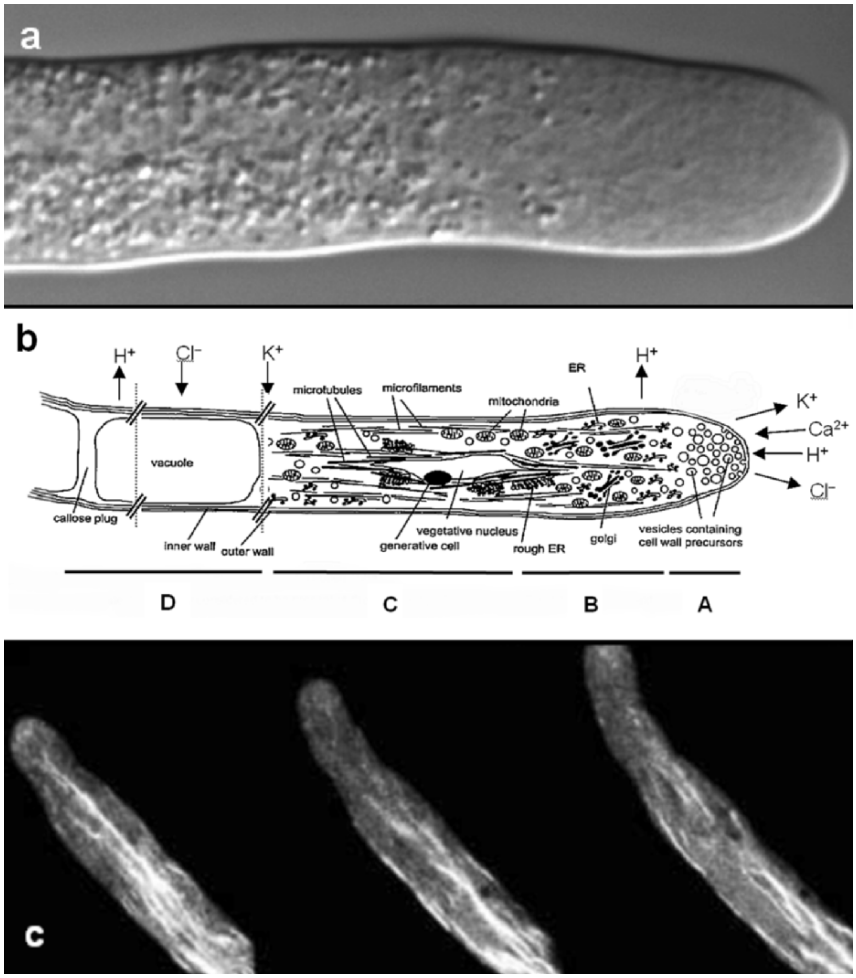

Fig. 2.2a DIC image of a *Lilium longiflorum* pollen tube (diameter~15 μm). The polarization of the growing tip is striking; the growing apex is devoid of large organelles and presents no organized movement (the "clear zone"). Behind, all the larger organelles are sorted out and move backwards in a fast, organized reverse-fountain streaming pattern. In this species, the tubes can grow in vitro as fast as 20 μm min^{-1}. **b** Diagrammatic representation of a pollen tube. *A–C* Cytoplasmic domains; *A* clear zone, *B* sub-apical domain, *C* nuclear domain; *D* vacuolar domain containing first callose plug (relative length of the domains is not drawn to proportion). *Arrows* represent ion fluxes known to be important in the establishment of polarity and pollen tube growth. **c** Time-course analysis of growth of a *Nicotiana tabacum* pollen tube transiently expressing a fusion protein construct of LAT52 (pollen-specific promoter), ADF (actin-depolymerizing factor) and GFP, imaged with two-photon microscopy. The three frames are separated by 30 s each. Growth of the tube is evident as well as the fast dynamics of the actin cytoskeleton in the formation and constant reshaping of the fibrils along the pollen tube. A finer meshwork of smaller filaments is present in the sub-apical area but this sequence was contrast-optimized to reveal the long actin cables promoting the vigorous streaming so characteristic of these cells (adapted from Boavida et al. 2005b)

pollen tubes must grow as fast as possible – bear in mind that in some species this amounts to several centimetres per hour (Barnabas and Fridvalszky 1984).

One of the easiest observable oscillatory features using video analysis of growing pollen tubes is growth rate. These oscillations appear to be spontaneous, since their occurrence has been reported in pollen tubes growing in minimal culture medium. They vary from species to species but, in *Lilium*, the first reported case of oscillatory behaviour (Weisenseel et al. 1975) and one of the most studied species, oscillations in growth rate appear only when pollen tubes reach lengths roughly above 0.6–1.0 mm. With shorter lengths, a full oscillatory behaviour has not been reported. Rather, the pollen tube emerges from the grain with a noisy and spiky growth rate, without any visible periodicity. Statistically, this has been defined as stable growth, with random fluctuations around a trendline until the final oscillatory growth phase is reached, with quasi-sinusoidal oscillations, three- to fourfold peak-to-trough variations and a period of 15–60 s (Feijó et al. 2001). However, in other species such as *Petunia* and *Nicotiana*, the picture is different, showing clearly pronounced oscillations persisting after germination (Geitmann et al. 1996). In fact, a closer examination of the fine growth characteristics of tobacco pollen tubes reveals more than only one, discernible oscillatory component (Fig. 2.3). In this specific experiment, owing to a frame acquisition exceeding five frames per second and, consequently, well above the level of the Nyquist criteria (computed from the theoretical resolution of a top-quality NA=1.4 objective lens and the average growth rate under these germination conditions), we can observe at least three different frequencies. One shows a short period of about 2.5 s, and corresponds to the raw data for growth rate (thin grey line). In addition, a moving average of 3 s applied to these data (thick black line) readily shows the most typical period observed in most species so far studied, which varies in the range 20–90 s depending on species/growing conditions (in this case – tobacco – roughly 1 min). Finally, in many instances, also a low-frequency oscillation of about 4–15 min occurs, particularly in tobacco and petunia (in this case, 4.5 min; this low-frequency oscillation corresponds to the "pulsatory" behaviour described by Geitmann et al. 1996). Since most of the available data correspond to the intermediate period (20–90 s), unless otherwise specified we here focus on these as characterizing the oscillatory behaviour of pollen tubes and corresponding to the central oscillator.

Are these growth oscillations important for pollen tube growth? When comparing average growth rates between pollen tubes for the different growth states reported, it is easy to understand that the shorter peaks defining the oscillatory period correspond to the highest average growth rate. It is therefore tempting to extrapolate that oscillations are a cause/consequence of the pollen tube growing as fast as possible, albeit limited by physical constraints in order to avoid cellular disruption.

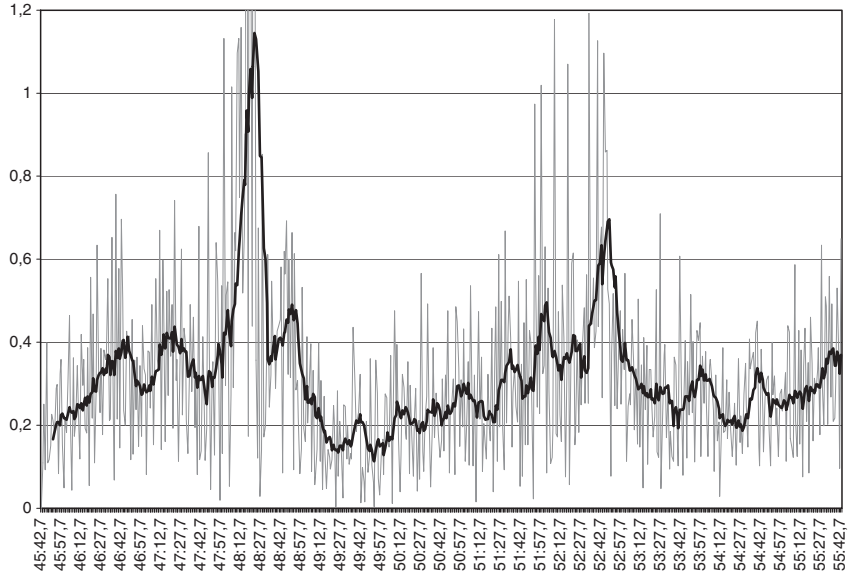

Fig. 2.3 Oscillatory growth characteristics of *Nicotiana tabacum* (tobacco) pollen tubes. A time-course movie was acquired using a PlanApo NA1.4, 60x Nikon lens with a Princeton Micromax 5 MHz camera, run by Metamorph at full acquisition speed. Frame rate was better than five frames per second. The sequence was then analyzed using the motion analysis functions of Metamorph, namely the "Track Object" algorithm, which assigns a geometrical centroid to a given selected zone and advances the centroid from frame to frame, thereby computing growth rate. In every attempt, localization of the centroid over the tip cell wall area provided the most accurate results when compared to frame-by-frame hand assignment of the tip edge. The software is calibrated with a micrometer and converts pixel changes into linear growth rate, which was then exported to Excel. Raw data (*thin grey line*) show an underlying oscillation which, with Fourier analysis, produced a peak with a period of about 2.5 s. A moving average of 3 s applied to these data (*thick black line*) highlights the major oscillatory component of the growth rate in this species, with a Fourier peak of about 57 s. Finally, there is a third low-frequency trend, with a period of about 4.5 min. This low-frequency oscillation is characterized by very pronounced amplitudes in growth rate. For that reason, it has become the easiest observable oscillatory pattern in non-computer-assisted processing of time-lapse movies of pollen tubes, especially in tobacco and petunia. This low frequency is apparently absent in lily, a reason why, before computer-assisted image processing became available, this species was considered to be non-oscillatory. Generally speaking, this low frequency seems to be associated with solid style species whereas less pronounced oscillations occur in hollow style species

2.4 Under Pressure

Chloride has often been associated to turgor pressure, because it has the capacity to be involved in salt extrusion, controlling the system osmolarity (White and Broadley 2001). In pollen tubes, chloride fluxes are in phase with growth oscillations and can exceed 1,000 pmol cm^{-2} s^{-1} (Zonia et al. 2001, 2002). This makes it very tempting to assume that turgor pressure is generally

ultimately controlled by chloride fluxes, as it is known to occur in other cells. However, less reasonable is the assumption that turgor might control growth, since the available data indicate that there is no correlation between these two parameters (Benkert et al. 1997). In the work of Benkert et al. (1997), pollen tubes were impaled and measured by means of a pressure probe, and an average of 0.2 MPa (or 2 atm) was determined to be necessary for growth. However, variations within one order of magnitude above that value were not reflected in any change in growth rate.

Nevertheless, one should not forget that pollen tubes are not homogeneous structures and, since they are highly polarised (in every sense of the word), the clear zone might have different physical properties. This could lead to localized changes in turgor which, due to some compensation mechanism, could be imperceptible elsewhere. Ultimately, such small variations in volume would lead to an infinitesimal decrease in magnitude which could be immediately compensated by cell wall and membrane elasticity, resulting in no measurable change. However, both of these situations would be plausible only if putative oscillations in turgor pressure were a consequence of tube growth, rather than a cause as has been proposed by other workers (Messerli and Robinson 2003). In proposing a causal role for turgor, Messerli and Robinson (2003) dismissed the data of Benkert et al. (1997) but, until today, have not justified this scepticism on the basis of other than formal circumstantial arguments, nor have they produced any meaningful alternative data on this aspect of turgor in pollen tubes.

On the other hand, turgor has been argued to play a role not only as a spatial constraint for tip growth morphogenesis but also in the penetration of the stigma and in the non-homogeneous cellular structure within the female organs, probably by providing the necessary rigidity for penetration and growth under differential pressure conditions (Money 2001).

2.5 Another Brick in the Cell Wall

"Without cell walls, plants would be pliant piles of PROTOPLASM, more like slime moulds . . ." (Cosgrove 2005), and pollen tubes surely would not exist. The rigid tubular structure is dependent on an extracellular wall capable of sustaining it and also plastic enough to enable pollen tube elongation (Steer and Steer 1989). Hence, the pollen tube cell wall has two different components: an outer one composed largely of pectin and cellulose, and an inner one with a sheath of callose (reviewed by Taylor and Hepler 1997). Cell wall precursors delivered at the tip are methyl-esterified pectins, preventing rigidification and sustaining a more flexible structure (Carpita and Gibeaut 1993). Callose and cellulose are absent from the tip (Li et al. 1994; Ferguson et al. 1998).

Still to clarify is the role of ions in forming the pollen tube cell wall. In other *Arabidopsis* tissues, it is known that some wall-associated kinases (WAKs) need calcium to bind pectins, which will then become part of the de-esterification process (Decreux and Messiaen 2005). However, these enzymes are absent in the pollen tube transcriptome (Becker et al. 2003; Pina et al. 2005), which does not invalidate another mechanism for calcium in this process. Protons are another possible candidate. Pectin methyl-esterases (PME) are present in pollen tubes (Li et al. 2002) and are thought to be responsible for the de-esterification process (Willats et al. 2001), releasing protons due to methoxyl-to-carboxyl conversion. Protons may act as regulator of the cell wall because some enzymes become active at lower pH (Nari et al. 1986; Wen et al. 1999). However, the pH decrease would not be at the apex but in the lateral flanks near it. Hence, lower pH would not prevent de-esterification at the tip, as it has been recently proposed (Bosch and Hepler 2005).

Independently or not of ionic interaction, in some species such as tobacco and petunia, arabinogalactans and pectins are deposited in ring-like structures with remarkable periodicity along the pollen tubes (Li et al. 1994). The frequency of this deposition can be correlated with growth rate variations and, if growth rate is slower, then an additional deposition of cell wall precursors can occur. This could be explained by a partial uncoupling between growth rate and cell wall deposition, i.e. growth rate varies but cell wall deposition or maturation occur at a uniform rate. In other species, such as *Lilium*, these events do not occur naturally but can nevertheless be induced experimentally (Li et al. 1996).

Rhamnogalacturonan II (RG II), and its cross-link with borate esters, seems to be important for the formation of the pectic network present in the plant cell wall (Matoh and Kobayashi 1998), since it is needed to dimerise this polysaccharide. Hence, it has been suggested that the boric acid present in most pollen germination media might play a role in stabilizing the pollen tube cell wall, even at extremely low concentrations. The effect on oscillations caused by cell wall stiffening can be explained by the presence of more dimeric RG II (Holdaway-Clarke et al. 2003), due to boric acid interaction.

2.6 Cytosolic Approaches to Oscillations: the Ions Within

Intracellular ion dynamics has been shown to play a central role in pollen tube growth and development. Cytosolic ion gradients seem to be strongly involved in the regulation of cytoplasmic asymmetry and in the maintenance of the growth axis in this type of cell, whereby calcium and protons are considered to be of major importance (Feijó et al. 1995, 2001; see Fig. 2.4). Cytosolic free calcium ($[Ca^{2+}]_i$) is a highly versatile, intracellular signalling molecule (Berridge et al. 2003) which is known to be present in pollen tubes

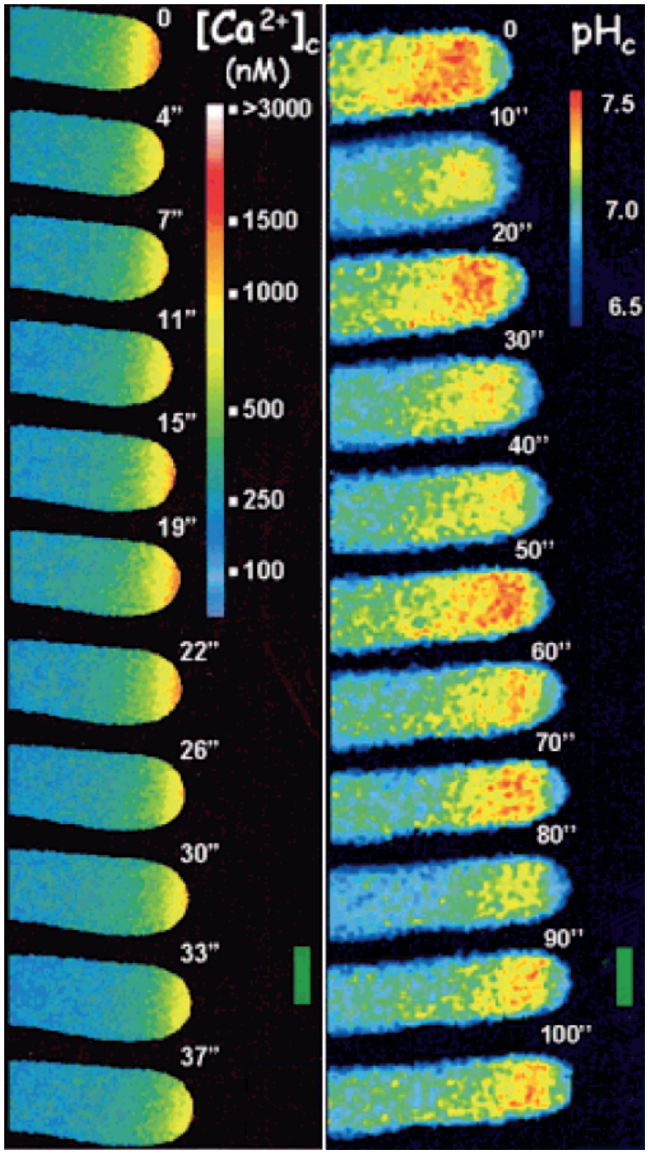

Fig. 2.4 $[Ca^{2+}]_i$ and pH_i oscillations in *Lilium* pollen tubes. *Left* $[Ca^{2+}]_i$ is elevated at the tip and can reach 10 µmol, oscillating almost in phase with growth with a lag of a few seconds. The peak-to-trough ratio varies by more than one order of magnitude. *Right* pH has an alkaline band at the shank, showing an acidic tip which can have a pH up to 6.5. The oscillations in protons produce a pH variation of 0.3 to 0.5 (adapted from Feijó et al. 2001)

in a non-ubiquitous fashion, forming a tip-focused gradient (Miller et al. 1992). Because the $[Ca^{2+}]_i$ pool at the apex oscillates (Pierson et al. 1996; Evans et al. 2001), and might participate in the vesicle fusion mechanism with the plasma membrane (Roy et al. 1999; Augustine 2001), it has been associated with delivering cell wall precursors and, consequently, is considered to support growth (Messerli and Robinson 1997). Furthermore, it has been experimentally verified that, every time there is a dissipation of the $[Ca^{2+}]_i$ gradient, the tube stops growing and, every time there is an arrest in growth, $[Ca^{2+}]_i$ is dissipated (Pierson et al. 1994). Another interesting result is that the higher $[Ca^{2+}]_i$ in specific spots at the tip seems to cause the pollen tube to turn in that direction (Malho and Trewavas 1996).

Calcium influx at the tip has been found to be necessary (Miller et al. 1992) and sufficient (Holdaway-Clarke et al. 1997) for maintaining a gradient reaching 10 μmol $[Ca^{2+}]_i$ in the immediate sub-membranar vicinity of the tube apex (Messerli et al. 2000). Despite a phase shift of more than 10 s relative to peak growth, it has been suggested that this influx is based on an increase in $[Ca^{2+}]_i$ and, consequently, growth (Hepler 1997). However, the issue of phase offset of $[Ca^{2+}]_i$ relative to growth is problematic because correlation analyses between the one and the other have been based on different methods. This has led to disparate results, from apparent synchronization in the studies of Messerli and Robinson (1997) and Holdaway-Clarke et al. (1997) to a 4-s delay in $[Ca^{2+}]_i$ elevation after peak growth rate in that of Messerli et al. (2000). It should be pointed out that the two earlier reports calculated growth rate from the intracellular fluorescence signal whereas the more recent analysis was based on wide-field differential interferential contrast (DIC) images. The former two presumably measured the extension of the cytosol (affected by the Poisson decay of fluorescence in the extreme) whereas the latter measured the cell wall refringence properties used by DIC. As depicted by a processed DIC sequence for a lily pollen tube shown in Fig. 2.5, the advance of the cytosol seems to actually precede that of the cell wall. This is a consequence of the cell wall thickness not remaining constant but also oscillating, presumably as a result of an offset between growth rate and vesicle secretion. Taken literally, the 4 s in $[Ca^{2+}]_i$ delay after the growth peak is probably a longer interval, which leaves a gap in the regulation of $[Ca^{2+}]_i$ oscillation at the tip. This finding, albeit counter-intuitive, can plausibly be explained by a cycle in which, when growth ceases or decreases, the putative calcium channels at the tip cease their activity and calcium uptake but the calcium levels are nevertheless maintained until the gradient is dissipated. This hypothesis is, however, tentative because of an almost complete lack of knowledge on the gating properties of any channel present in pollen tubes (although Dutta and Robinson 2004 have described a putative stretch-activated calcium channel in pollen tube protoplasts, these data have not yet been confirmed by other groups. Given the contentious nature of this type of channel and doubts about channel characterization, this study is unlikely to be the final word on calcium transport into pollen tubes). Adding to this the

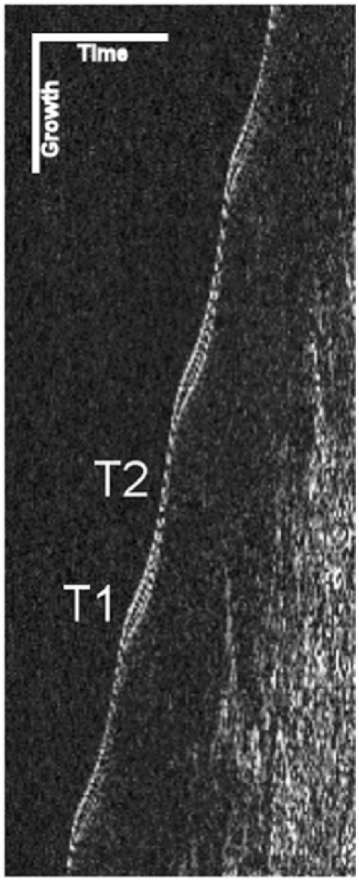

Fig. 2.5 Kymograph showing membrane and cell wall interfaces from a DIC time-lapse sequence of a growing pollen tube after a Prewitt detect edges filter (image processing algorithm which highlights the limits of defined optical objects). The kymograph depicts the edges of the plasma membrane and cell wall variations over time, by stacking one line per frame over the "time" direction. Each of these lines represents a transect along the central length of the pollen, and the slope of both the membrane and cell wall in the "growth" direction is a direct consequence of growth. Humps on the slope of advance reflect the oscillatory nature of growth. As can be observed, the plasma membrane (*inner curve* relative to the tube content) is the first to reduce and to increase speed. The cell wall presents some relative inertia in terms of growth (*T1*). After the membrane starts to reduce speed, the tube continues with the same measured increment, since the cell wall continues to expand. DIC image speed measurements are based mostly on cell wall refringence properties. Note that there is a certain time point (*T2*) at which both are growing at the same constant speed. This result is consistent with the need for a certain threshold of $[Ca^{2+}]_i$ for maximizing vesicle fusion but thereafter is independent of concentration (horizontal bar, 10 min; vertical bar, 10 μm)

fact that, both in the fluorescence and in the DIC analyses of growth, there are many different standards used in imaging by various groups, it is evident that the relative phase shifts between growth and $[Ca^{2+}]_i$ should still be considered a matter of debate. As a final example, based on our own experience, image processing of sequences acquired with a lower NA, dry DIC lens (e.g. 20x) produces phase shifts different than when using a higher-quality oil-immersion lens also with DIC (e.g. NA=1.4, 40x oil immersion), which has revealed that chloride effluxes are essentially in phase with growth rate (Zonia et al. 2002).

Since water ionises spontaneously and is the major component of living organisms, protons are probably the most basic and common second messenger in several cellular mechanisms. They are know to be involved in modulating enzymatic activity (Guern et al. 1991), phosphorylation (Blowers and Trewavas 1989), cytoskeleton activity (Yonezawa et al. 1985; Beaulieu et al. 2005), endo/exocytosis (Cosson et al. 1989; Smith et al. 2002) and energetic cycles (Sanders and Slayman 1982). Hence, the pH inside cells has to be tightly regulated, especially in those such as pollen tubes in which spatial dynamics plays such a major role.

Evidence of an oscillatory-like behaviour shows pH to be an important factor controlling growth oscillations (Messerli and Robinson 1998). Furthermore, by using a more sensitive imaging setup, it was possible to demonstrate that the tube shows an acidic domain apressed to the membrane apex, which exists only when the tube is growing, and a sub-apical alkaline area of magnitude apparently oscillating in reverse to the growth phase (Feijó et al. 1999). The exact role of this gradient is still far from having been clarified but possible links lie in the control of calcium channels (Y. Chen et al. 1996), or the localization and rates of endo and/or exocytosis (Davoust et al. 1987; Smith et al. 2002) or actin cytoskeleton modulation via pH-dependent actin proteins such as the actin-depolymerizing factor (ADF; Chen et al. 2003).

Other ions are also likely to contribute to our knowledge of the dynamical regulation of pollen tube growth but there is presently a complete lack of information on this topic. Specifically, the use of genetic probes is still on its infancy and restricted to $[Ca^{2+}]_i$ (Iwano et al. 2004; Watahiki et al. 2004). Nevertheless, novel imaging methods have opened new possibilities for better and more accurate reanalysis of all these data (Feijó and Moreno 2004; Moreno et al. 2006).

2.7 On the Outside: Ions and Fluxes

More than three decades ago, Weisenseel et al. (1975) demonstrated that pollen tubes are a living electric dipole, with an inward electric current throughout the tube plasma membrane, and an equilibrating outward current on the pollen grain. These authors were also the first to realise that this current changed from a steady state to show a pulsating behaviour as pollen

tubes reached a certain length, this continuing until the death of the cell. The formation of this cellular dipole highlights the importance of ionic fluxes, and its study has become a priority to better understand pollen tube growth and development.

Earlier studies were, however, handicapped by the original design of the "vibrating probe" used at the time – a wire electrode, capable of measuring voltage differences alone, without any possibility of ionic discrimination. On top, the electrode was vibrated at elevated frequencies (hundreds of Hz), prone to produce artefacts by destroying slow-forming ion gradients. This original approach subsequently gave place to the use of ion-selective vibrating electrodes (Kuhtreiber and Jaffe 1990) which, on the one hand, are ion-specific on the other hand, are operated at much lower frequencies, thus being much more non-invasive. Ion-selective vibrating probes gave the possibility to access both the intensity and direction of ionic fluxes across the membrane, and also at specific points of the cell, even when growing fast (reviewed by Shipley and Feijó 1999, and Kunkel et al. 2006). Contrary to what was initially described by using the original voltage vibrating probe and substitution experiments (Weisenseel and Jaffe 1976), the use of ion-specific probes showed that Ca^{2+} is a major current carrier in the oscillatory phase (Holdaway-Clarke et al. 1997), with important contributions of K^+, H^+ and Cl^- as well (Feijó et al. 1999; Messerli et al. 1999; Zonia et al. 2001, 2002; reviewed by Holdaway-Clarke and Hepler 2003).

The study of calcium influx in the tip has revealed a clearly oscillatory behaviour (Pierson et al. 1996). These fluxes exhibited the same period as did growth but were out of phase, lagging 11–15 s behind the growth peaks (Holdaway-Clarke et al. 1997; Feijó 1999; Messerli et al. 1999). Hence, physical growth is a possible modulator of the influx profile.

As previously stated by Holdaway-Clarke et al. (1997, 2003), there is sufficient influx of Ca^{2+} at the tip entering into the protoplast to support the intracellular gradient oscillations observed and, therefore, it would seem logical if both variables were to be in phase. According to these authors, however, the cell wall matrix may act as a buffer for the entry of Ca^{2+} into the cytosol, due to the binding of this ion to carboxyl groups which appear unbound in the newly formed cell wall (Li et al. 1994). The specific kinetics of this binding would then be considered an explanation for the reported delay between the intra- and extracellular ionic peak of oscillations relative to growth. This theory does not take into consideration another possibility set forth by Carpita and Gibeau (1993), who proposed that the reaction between Ca^{2+} and the acidic residues in homogalacturonan pectin and cross-link adjacent chains in the cell wall would confer rigidity to it. This appears not to occur, however, because growth rate does not seem to be affected as it should be if this were the case. This aspect thus remains open to discussion.

Feijó (1999) and Messerli et al. (1999), also working with ion-selective vibrating probes on *Lilium*, reported proton influxes near the tip with a slightly smaller lag relative to growth rate (6–9 s). Based on the cell wall

buffering model discussed above, protons might be involved in mechanisms affecting cell wall rigidity, which could be responsible for this delay due to proton retention. Again, the hypothesis awaits experimental confirmation.

Messerli et al. (1999) also found that potassium (K^+) influx measurements showed a similar phase and magnitude as those of the proton influx measurements. It should be pointed out, however, that the extremely high values reported for the K^+ fluxes would have created non-trivial problems in terms of membrane potential and turgor maintenance. These values have not yet been confirmed by other laboratories, an important point because the ionophore cocktails usually utilized for measuring K^+ are far less reliable than their counterparts for Ca^{2+} and H^+. Still, these authors have suggested a mechanism based on cationic co-transport or resulting from small changes in turgor pressure, after each growth pulse (Messerli et al. 1999). To date, the evidence for both remains completely circumstantial.

A curious fact regarding chloride dynamics is that, compared to Ca^{2+} and H^+, the fluxes occur in the reverse direction. In lily pollen tubes, chloride influx takes place in the more basal regions, at least 12 μm from the tip, and the efflux site is the apical region of the pollen tube. Vibrating probe measurements of chloride effluxes have shown the existence of an oscillatory behaviour for pollen tubes of tobacco and, above 600–900 μm, of lily. The disruption of the chloride fluxes (by means of channel blockers) showed chloride flux dynamics to be vital for pollen tube growth, and evidence exists of its role in hydrodynamics control. Since a phase correlation was observed between oscillation growth rate and chloride efflux, a relationship between chloride efflux and vesicle trafficking towards the apex was also suggested (Zonia et al. 2002).

Basically reproducing these results both in terms of spatial distribution and chloride-equivalent fluxes, Messerli et al. (2004) have recently questioned the reliability of these data, on the basis of the lack of specificity of the chloride ionophore used in the electrodes. The lack of specificity for different anions was indeed reported and appropriately dealt with by means of various controls by Zonia et al. (2002). Admittedly, the chloride fluxes reported could still have been affected by the interference of other anions. Under all the controlled conditions, however, these should have been minimal and, hence, the reported fluxes should have been constituted primarily by chloride. Messerli et al. (2004) also contested the authors' conclusions on the grounds of (1) the putative interference of proton gradients on the chloride ionophore, i.e. by interference of the MES buffer, and (2) the alleged absence of chloride channels in pollen tubes, as detected by patch-clamp (Dutta and Robinson 2004). Unfortunately, the first argument is based on incomplete data and biased modifications in basic experimental procedures which impaired the desired reproduction of the Zonia et al. (2002) experimental setup – meanwhile, this setup has been confirmed to be correct and meaningful (Moreno, Colaço and Feijó, unpublished data). The second argument seems remarkable based even on only minimal knowledge of plant physiology, namely that a plant cell can

exist without specific chloride channels in the cell membrane. For the record, the transcriptome analysis of *Arabidopsis* pollen (Becker et al. 2003; Pina et al. 2005) shows the presence of, among others, two pollen-specific expressed chloride channels (clc-c- and clc-e), both with elevated levels of expression, and a number of other putative ABC proteins which, more than likely, are active in chloride transport. Why these genes would be specifically and highly expressed in pollen if not to form active membrane channels would definitely constitute a far greater mystery than Dutta and Robinson (2004) not finding these by patch-clamping pollen protoplasts.

In animal cells, chloride is known to be involved in the control of cellular volume (X.H. Chen et al. 1996) and, in plants, in turgor regulation (Shabala et al. 2000), possibly in pollen tubes, too. However, further studies on the importance of oscillatory ion fluxes for growth are needed in order to completely understand their role in the pollen tube as cell, and their participation in the mechanistics of its underlying oscillator.

2.8 Actin Cytoskeleton: Pushing it to the Limit

In pollen tubes, the cytoskeleton performs at least three central functions: enabling vesicles to reach the apical zone for exocytosis, cytoplasmic streaming, and the transport of gametes (Cai et al. 2005). Hence, unravelling the role of cytoskeleton dynamics in pollen tube growth and, consequently, in oscillations is of major importance for our understanding of the other components too.

By freezing the cytoskeleton by means of cytocalasin D, vesicles are precluded from reaching the tip for exocytosis, and growth does not occur (Picton and Steer 1981). By exposing pollen tubes to sub-vital concentrations of lantranculin B, which blocks actin polymerization, oscillations are abolished (Vidali et al. 2001) and growth rate decreases slightly, suggesting that actin polymerization has an active role in controlling pollen tube oscillations, which is consistent with vesicle oscillations at the tube apex (Parton et al. 2001). More recently, the actin-stabilizing drug Jasplakinolide was also shown to have this effect (Cardenas et al. 2005), highlighting the fact that growth can be sustained even under conditions in which oscillations are below the resolution threshold of current methods, by affecting cytoskeleton dynamics through either de-polymerization (latB) or stabilization (JASP).

That tip-localized F-actin microfilaments oscillate in phase differences of 15–30 s preceding growth in tobacco pollen (Fu et al. 2001) may be an indication of a stronger involvement of actin in this process. However, actin is controlled by, among other factors, Rho GTPases (Fukata et al. 2003).

Recent work suggests the activity of ROP1, a plant-specific *Rho*-related GTPase, as one possible control mechanism not only for actin microfilament but also for growth rate and cytosolic Ca^{2+} oscillations (Hwang et al. 2005). The Rho switch is very straightforward but, for example, in humans, it has

over 60 positive and over 70 negative modulators. Despite a simple mechanism, it is very tightly regulated and involved in numerous processes (Etienne-Manneville and Hall 2002).

In pollen tubes, ROP1 is located in the cytosol but shows up in the apical membrane in the tip region as well (Kost et al. 1999). It seems to activate at least two downstream pathways mediated by two downstream target proteins, RIC3 and RIC4; these, in turn, seem to be involved in the formation of the tip-focused $[Ca^{2+}]_i$ gradient and the assembly of apical actin respectively (Gu et al. 2005). Despite not being in direct competitive binding to ROP1, because they are localized differentially in the cell, the fact that RIC3 and RIC4 act as antagonists can therefore be explained by the interaction with their downstream targets, $[Ca^{2+}]_i$ and actin, suggesting their existence as a self-regulatory system.

ROP1 activity, described through the fusion protein RIC4::GFP, has been shown to oscillate and, when submitted to correlation analysis with growth rate, peaks of activity have been demonstrated to occur before peaks of growth rate, in phase with f-actin tip assemblies. ROP1 is present at the tip in the plasma, in an asymmetric distribution "anticipating" direction changes in tubes, which suggests some kind of regulatory activity (Hwang et al. 2005).

RIC3 activity, involved in cytosolic calcium oscillations, usually lags after the growth peaks. Since ROP1 activates both RIC3 and RIC4 pathways, how can one response appear before the growth peak and the other afterwards? Gu et al. suggest that the RIC3 pathway is longer and more complex than the RIC4 pathway, consistent with the temporal gap between responses. A role for cytosolic calcium-controlling ROP activity, probably through cycling between GTP-(active) and GDP-(inactive) bound forms of ROP, has been suggested but not shown (Gu et al. 2005).

2.9 Membrane Trafficking and Signalling on the Road

In nature, pollen tube development it is not an independent cellular process but rather a result of pollen–stigma interactions which direct pollen tube growth through physical or chemical cues. The membrane plays a very important role in this respect because it is in contact with both the intra- and extracellular milieu. Despite some data showing that oscillations occur also at least under semi-vivo conditions (Iwano et al. 2004), there is ample evidence that pollen tubes oscillate under in vitro conditions, proving that the cell itself works as an oscillator (Feijó 1999; Feijó et al. 2001), without needing external factors. However, we do not know how pollen–stigma interactions could affect and possibly modulate this pulsed growth.

If the membrane, as an interface with the exterior, is not critical for this behaviour, the way it is deposited and recycled should be a fundamental process for oscillations, because vesicles have to interact with cell wall precursors in a way which should be compatible with non-linear growth.

By imaging endogenous membrane dynamics using amphiphilic dyes such as FM, Parton et al. (2001) showed that the pool of vesicles in the inverted cone area oscillates and this oscillation precedes growth peaks. Even more relevant, in a second set of experiments, these authors provided the only published evidence that oscillations may continue even when pollen tube growth has stopped, since they showed an oscillation in membrane trafficking as long as intracellular streaming continued, independently of growth (Parton et al. 2003). According to these authors, by inhibiting vesicle trafficking by means of brefeldin A, it was possible to demonstrate an induced membrane body oscillating with a frequency five times higher than the normal growth rate oscillations. This could probably be an effect of the BFA treatment but the principle of having a mechanism oscillating without the intracellular calcium gradient, secretion and apical extension or growth proves that there are several low-level intrinsic oscillation machineries which seem to be able to sustain some free-running properties independently of growth. Despite the fact that some calcium oscillations were found in non-growing pollen tubes (Messerli and Robinson 2003), secretion was probably occurring. In fact, by increasing extracellular osmolarity, which decreases turgor pressure, the tube shrinks and detaches from the cell wall, and another layer of cell wall differentiates. Decreasing the pressure even further, this second layer becomes clear (Moreno, Colaço and Feijó, unpublished data).

The other major role of the membrane involves phospholipid signals (Meijer and Munnik 2003). Recent studies have shown that phospholipase C in petunia (Pet PLC1) is present in the membrane and cytosol of pollen tubes (Dowd et al. 2006). This enzyme activity produces IP3, which might work as a second messenger to release calcium from intracellular stores, but this is probably a simplistic description of a more complex mechanism (Holdaway-Clarke and Hepler 2003). Another path proposed by Dowd et al. is that the PLC substrate PtdInsP2 acts per se as a tip growth regulator, since they observed that growing and non-growing tubes present a different localization pattern. In non-growing pollen tubes, this substrate was localized homogeneously in the membrane and, in growing pollen tubes, it was present as an increasing gradient to the apex. This is consistent with actin dynamics once it binds to ADFs (Chen et al. 2003).

The role of the endo-membranar system in the regulatory mechanism of pollen tube oscillations remains to be investigated. Despite differences with mammals (Battey et al. 1999), Ras-related small G-proteins are known to be an essential key for membrane organization (Zerial and McBride 2001). In pollen tubes, Rab2 regulates the ER-to-Golgi network (Cheung et al. 2002), and this process is fundamental for tube elongation. Thus, studying oscillations in occurrence of these GTPases would be important for understanding membrane regulation and oscillations in pollen tubes. As an example, a constitutive active form of Rab11 produces pollen tubes which grow in zigzag with a consistent pattern (de Graaf et al. 2005), suggesting that it might have a role in controlling some sort of spatially oscillatory pattern.

2.10 Conclusions

Three criteria are deemed as fundamental for the study of oscillatory behaviours in living systems: the system should be studied with a minimum amount of interference, the existence and consecutive study of several independent variables relevant to the description of the system should be done continuously and, finally, a synchronization of these variables in time should exist (Feijó et al. 2001). Growing pollen tubes can satisfy, within certain limits, the above conditions, making these an exciting and useful model for non-equilibrium systems.

The fact that several variables display an oscillatory behaviour in pollen tubes does not imply that all have a direct, active role in growth oscillations. Indeed, viewing the system as a whole, it is not straightforward to understand which possible set of components forms the major motor for the initial oscillatory effect. In non-oscillatory phases, some cells already possess native oscillators (Gilbert and Lloyd 2000) but these remain to be fully determined, since they are probably being ruled by a chaotic behaviour. It would be crucial to know which factors are involved in this synchronization, since this would give some insight about the starting point and probably the most important factors for this dynamic behaviour.

Using differential interferential contrast images, it is easy to measure growth using pattern recognition software, which traces tip growth rate. Hence, it is possible to collect very precise data and to establish which components might be involved and how these affect growth. However, with more sophisticated tools such as wavelet analysis, it might be possible to unveil other frequencies which can not be identified by Fourier analysis, in which the number of time points is too low for this type of approach. Nevertheless, it is still unknown if pollen tubes have one or more oscillatory subsystems, and how each control factor may influence the whole system. Such measurements will have to be done in a way that external factors can be absolutely minimised, to avoid external triggering of any kind, and data representation must be optimised (Gilbert and Ferreira 2000).

Since the ultradian rhythms present in pollen tubes are not temporally coincident with protein synthesis and degradation, a simpler mechanism might be triggering the overall oscillation. This does not necessarily mean that other cellular components can not modulate or influence this. To date, the strongest candidate seems to be the membrane trafficking machinery, since this is the only mechanism which oscillates independently of tube extension (Parton et al. 2003). However, one cannot ignore the fact that blocking actin polymerization abolishes oscillations but not cellular growth (Vidali et al. 2001), and actin is, at least partially, controlled by Rho GTPases (Hwang et al. 2005).

Cytosolic free calcium is always present as a tip-focused gradient during the tube extension process, and might also work as part of the circuit as a messenger for certain states in the cell. The fact that it does not seem to have

a direct correlation with growth suggests that it works rather as a switch. After a certain threshold, we have maximum exocytosis but such high levels of calcium at the apex seem to have other roles as well. This threshold explains why the peak growth phase can occur only seconds before calcium peaks, with no difference in tube growth rate.

Despite the fact that several oscillator components are known, the interactions between these are basically not understood, which leaves us far from understanding the whole picture about the biophysical and biochemical components which make this specialized cell achieve such a high dynamics. If it is true that it oscillates *in vitro* independently of other cells or tissues, it is also too simplistic to consider these oscillations as a way to control cell growth by itself. The ionic currents generated by oscillating pollen tubes have peaks of higher magnitude than is the case for steady-state growth currents, and can be used as signalling cues to the surrounding medium. To date, the difficulty related with imaging pollen tubes in vivo do not allow us an accurate measurement of growth and under which circumstances this aspect is important.

Considering that, as cells, pollen tubes have a crucial, yet very basic function and are morphologically very simple, it is important to develop theoretical models capable not only of explaining the oscillating properties of the system but also of providing a link to its relevance in growth and morphogenesis. The fact that the rhythm is imposed in vitro by the cell itself highlights that oscillations are the natural way for cells to develop and to achieve their goals, independently of a measurable rhythm, which can help to understand other general mechanisms in cellular physiology.

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3 Ultradian Growth Oscillations in Organs: Physiological Signal or Noise?

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Abstract

This review examines ultradian oscillatory growth in the multicellular organs of higher plants. My objective is to derive insight about the underlying physiological processes powering expansion. If the process of diffuse growth is inherently oscillatory, then it is reasonable to expect entrainment of these cellular oscillators across a tissue and the emergence of coherent macroscopic growth oscillations. After reviewing studies of circumnutation and linear growth, it appears that such entrainment is rare or weak. I argue that rather than reflecting the existence of an inherent oscillation in the process of diffuse growth, ultradian movements of plant organs reflect successive responses to mechanical perturbation.

3.1 Introduction

3.1.1 Oscillations as Window into Growth

A growing plant organ comprises thousands of cells. These cells have different shapes, sizes, and states of differentiation. Despite this, the growth of plant organs is coherent, meaning that each cell grows essentially as its neighbor does. How is such uniformity of growth achieved? The cell wall provides a mechanical framework that can constrain the expansion behavior of individual cells by virtue of its continuity. However, cells are able to exert a considerable control over their growth locally, as seen in bulliform cells, trichomes root hairs, and even tropic bending. A common, limiting cell wall is presumably not enough to synchronize growth among a thousand neighboring cells.

An answer is offered theoretically by oscillations. Oscillatory behavior commonly characterizes complex, cellular processes, such as glycolysis or division (Goldbeter 1996). Expansion of a cell is also a complex cellular

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process, comprising steps that could be linked with delayed feedback, a condition for the emergence of a stable oscillation. These steps include water uptake, secretion, incorporation of material into the cell wall, and irreversible (i.e., plastic) as well as reversible (i.e., elastic) deformation of cell wall structure. As an illustration of how expansion could be oscillatory, suppose water uptake were linked to turgor loss, such that aquaporins would open once irreversible (plastic) deformation of the cell wall had decreased turgor sufficiently; the influx of water would raise turgor and hence close the water channels, not to open again until continued plastic deformation had again decreased turgor sufficiently (Fig. 3.1). This hypothetical loop illustrates feedback between steps in the growth process, and many other such loops could be imagined. To the extent that the feedback is delayed, an oscillation becomes stable. When neighboring, individual oscillators share input or output, they are easily synchronized (Goldbeter 1996). Cells of a growing organ have common cell walls and share water; therefore, it is plausible that an organ synchronizes cellular growth oscillations.

This review will examine oscillatory growth behavior. My objective is to derive insight about the underlying physiological processes powering expansion. I will not treat oscillations that are circadian because these are likely to be linked to diurnal rhythms of whole-plant performance, rather than to

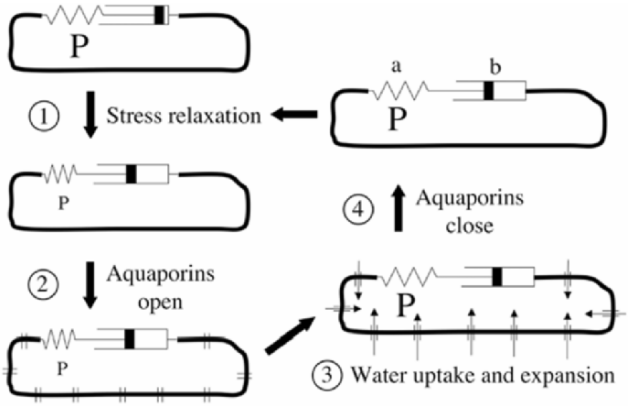

Fig. 3.1 Hypothetical model of diffuse growth giving rise to oscillatory expansion. For a single cell, the mechanical behavior of the cell wall is idealized by an elastic element (*spring*; *a*) and a plastic element (*dash-pot*; *b*) in series. Turgor pressure (*P*) is held by the deformation of the elastic element. Step *1*: the elastic element contracts, doing work against the plastic element. This lowers turgor. Step *2*: the cell senses lowered turgor and opens water channels (aquaporins). Step *3*: the open water channels allow water to move into the cell down the water potential gradient. This rapid water entry stretches the elastic element, thus enlarging the cell's volume and restoring turgor. Step *4*: the cell senses restored turgor and closes the aquaporins. The cell then undergoes stress relaxation and the cycle repeats. In a tissue, cell walls are shared and water supply is channeled; therefore, such an oscillation, in principle, could become synchronous over the tissue

growth itself. Also, I will not treat growth oscillations in single cells (the interested reader may consult the review in this volume by Moreno et al., Chap. 2), even though my objective is exemplified beautifully by Castle (1940) who detected an oscillation in the rotary movement of a single-celled, fungal sporangiophore, and argued from the oscillation's amplitude that expansion depends on the discrete insertion into the cell wall of a 7-nanometer brick every 200 milliseconds.

3.1.2 Growth Versus Movement

Unfortunately, the word *growth* is used in two distinct ways. On one hand, the length of an entire organ may be measured over time and its rate of increase called a *growth rate*; an equivalent rate is obtained by measuring the position over time of the tip of the organ. On the other hand, a growth rate can refer to relative expansion, often reaching to the level of a single cell, or indeed to the elemental deformation of a unit area of cell wall. The latter is the output of the growth mechanism whereas tip displacement integrates the expansion of the entire growth zone, often many centimeters long and containing cells at different developmental stages. Therefore, the length of an organ, or the position of its tip over time, provides limited information about cellular machinery. For clarity, I will refer to the rate of displacement of an organ tip as a *velocity*, characterizing data of that kind as referring to movement; in contrast, I will use *growth* to denote relative expansion, preferably close to, if not actually on, the cellular scale. Oscillations in movement can provide insight into growth mechanisms, but care must be taken because movement reflects cellular expansion indirectly.

3.2 Circumnutation: Growing Around in Circles?

If oscillatory growth behavior among individual cells is entrained, then organs should be characterized by macroscopic growth oscillations. This is believed to be true because it is assumed, first, that the stems and roots of essentially all plants undergo oscillatory movements called circumnutation and, second, that circumnutation is a coherent growth oscillation. Both of these assumptions need to be examined.

The stem tips of some plants undoubtedly move in circles or ellipses with large amplitudes and regular periods for many days (Baillaud 1962). This behavior reflects an obvious circumnutation, clearly adaptive for vines and climbing plants, but happening also in species such as sunflower (*Helianthus annuus*) where synchronization of growth among cells is as good a reason as any other for the behavior. However, the tips of stems and roots of other plants move in erratic trajectories with small amplitude and period far less

regular than that of twining plants (Heathcote and Idle 1965; Spurný et al. 1978; Barlow et al. 1994; Schuster and Engelmann 1997). To claim that all plant organs circumnutate is to assert that the large and regular movements of the French bean (*Phaseolus vulgaris*) stem (Millet and Koukkari 1990) are the same as the tiny and erratic ones of a grass rhizome (Fisher 1964). To be prudent, we should learn more about the mechanism of each type of movement before equating them.

Whether large and regular or small and erratic, circumnutations are widely ascribed to differential growth. But this need not be the case. In many plants, movements of leaves have periods of an hour or two, similar to circumnutations, and are powered by a specialized group of cells, the pulvinus, encircling the petiole at its base: the petiole lifts when adaxial pulvinar cells contract and abaxial cells expand; it lowers when the reverse happens (Satter 1979). The pulvinus moves the leaf by equal increases and decreases in cellular volume on each side, without any net change in volume. Therefore, these leaf movements are reversible and independent of growth.

As if a pulvinus were spread throughout the bending stem, reversible volume changes have been implicated in circumnutation. Pine (*Pinus sylvestris*) hypocotyls continue to circumnutate for a few periods following decapitation and the cessation of net elongation (Spurný 1975). In a tour de force, measurements of the growth of circumnutating French bean stems showed that most of the bending stem enlarges and contracts reversibly, conceptually like a pulvinus (Caré et al. 1998). Consistently, the bending part of the French bean stem undergoes alternating changes in cell length, turgor, ionic composition, and water permeability, reminiscent of those that occur in pulvini (Millet et al. 1988; Badot et al. 1990; Comparot et al. 2000).

That circumnutation can be powered by reversible changes in volume in the manner of a pulvinus has several consequences. For one, it means that a supposed universal habit of plants to circumnutate cannot be taken to imply an equally universal tendency to growth oscillations. In addition, a major topic of research on circumnutation has been to determine to what extent this movement can be explained by gravitropism. This explanation, formulated into an explicit model 40 years ago (Israelsson and Johnsson 1967), is that a stem responds gravitropically, overshoots its target angle, bends again, and overshoots again, thus creating an oscillation. Although the occurrence of circumnutation in space flight where gravitational force is all but absent has shown that gravitropism is not essential for circumnutation (Brown et al. 1990), the relevance of gravitropic overshoot for the oscillatory movements of stems and roots continues to be debated (Johnsson 1997; Hatakeda et al. 2003). However, gravitropic bending is accepted as being based on differential growth. If so, then those circumnutations powered by a diffuse pulvinus can be, for that reason alone, distinguished from gravitropism mechanistically.

Although circumnutation can involve reversible oscillations in cellular volume, it can also involve growth oscillations. Growth rate oscillations, 180° out of phase on either side of the circumnutating stem, occur in the epicotyl

of Alaska pea (*Pisum sativum*; Baskin 1986) as well as in the sunflower hypocotyl (Berg and Peacock 1992; Fig. 3.2). In both species, the seedling shoot undergoes more or less linear circumnutation, allowing growth to be measured with a single camera. Although Baskin (1986) measured the expansion of 1-cm-long zones and could have missed some contraction, Berg and Peacock (1992) measured 2-mm zones, and found that differential expansion is responsible for most of the bending (Fig. 3.2). Interestingly, these authors did record negative elemental elongation rates; hence, contractions may contribute to the oscillation. While the presence of a large contraction, as seen for the French bean, demonstrates a reversible, non-growth process, even a total absence of contraction cannot exclude a contribution from elastic behavior because reversible as well as irreversible processes are superimposed within the wall (Proseus et al. 1999; Fig. 3.1). Given the difficulty of measuring growth for large-amplitude circumnutation, the relative prevalence of diffuse

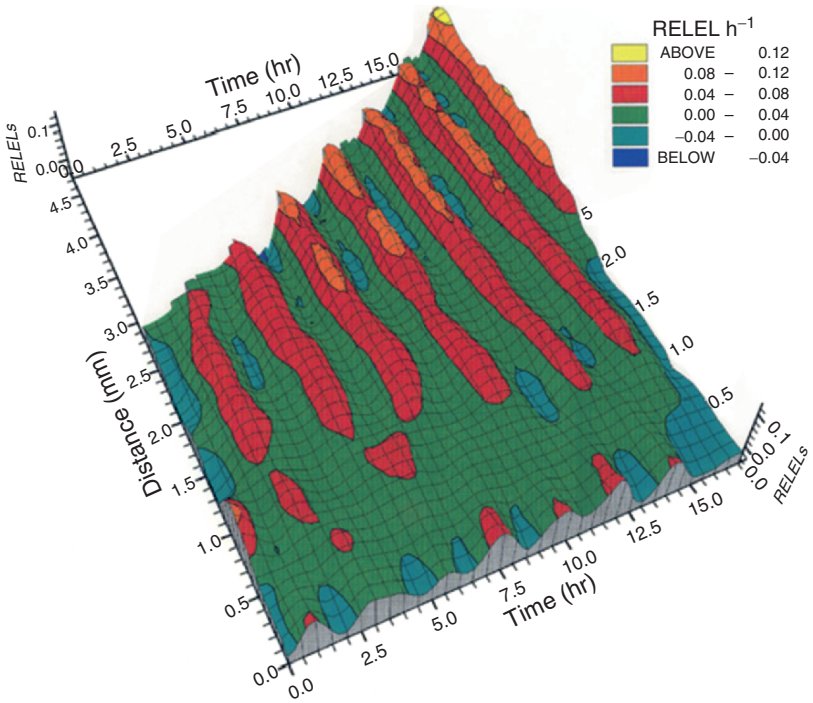

Fig. 3.2 Three-dimensional plot of displacement and elemental elongation versus time for a circumnutating sunflower hypocotyl (modified from Berg and Peacock 1992). The hypocotyl was marked at 2-mm intervals and growth rates recovered from marks digitized at 15-min intervals. Data were interpolated to generate smooth contours. The figure shows one side of the hypocotyl: the other side resembles this but is out of phase by 180°. The peaks of elemental elongation rate occur almost synchronously along the hypocotyl or move rapidly toward the base, and the troughs attain significant negative values (contraction) in the apical part of the organ

pulvini, as in French bean, and out-of-phase growth oscillations, as in pea and sunflower, is likely to remain unknown for a considerable time.

When species with thin roots, such as arabidopsis (*Arabidopsis thaliana*), are grown on a surface that is between horizontal and vertical, the root will grow in a sinusoidal pattern that has been attributed to circumnutation ("root waves"; Simmons et al. 1995) and a regular growth oscillation has been presumed. However, in an elegant analysis, Thompson and Holbrook (2004) showed that the undulating pattern represents buckling of the root, and results from gravitropism and friction between the root tip and the substrate. No oscillation in growth is occurring. The tip displacement rate of the root fluctuates erratically. This illustrates how a regular, oscillatory pattern in the shape of the root can be built up without an oscillation in growth rate. Interestingly, the amplitude of root waves varies among rice (*Oryza sativa*) accessions and is correlated with seedling establishment on flooded soil (Inoue et al. 1999), an observation that links the interplay of gravitropism and mechanical responsiveness to successful root penetration. But spiral waves in thin-rooted species cannot be cited in support of the prevalence of growth oscillations.

3.3 In Search of Ultradian Growth Oscillations

Under the hypothesis that expansion in plant cells is inherently oscillatory, and hence readily entrained among the many growing cells of an organ, the emergent, master oscillation might most simply be expected to occur symmetrically, rather than as a traveling wave rotating around the circumference. Note that a model for a traveling growth wave has been developed for the lateral movements of maize roots (Shabala and Newman 1997b). Symmetrical entrainment would give rise to oscillations in tip displacement velocity but not to oscillatory lateral movement. I will call these *linear* oscillations, because they are in line with the longitudinal axis of the organ. However, as the symmetry might not be perfect, oscillatory lateral displacements of small amplitude might plausibly indicate synchronized linear oscillations. How prevalent are well-synchronized linear oscillations?

Over the years, the linear displacement of a variety of plant organs, shoots, roots, and leaves has been followed at high resolution and over several hours or even days. Fluctuations are usually but not always reported. According to one of the first botanical applications of position transducers, lupine (*Lupinus perrenis*) stems, intact or excised, grow smoothly (Penny et al. 1974), and high-resolution photographic analysis of elemental elongation of intact, red-light-grown maize coleoptiles failed to find oscillations (Baskin et al. 1985). Nevertheless, fluctuations in growth velocity are common, whether detected with position transducers (e.g., Behringer et al. 1990; Yang et al. 1993) or with optical means that do not involve contact with the organ (Jiang and Staude 1989; Liptay et al. 1995).

In roots, various types of movements have been recorded, almost always with small amplitudes, wandering trajectories, and poorly defined periods, ranging from minutes to hours (Spurný 1966; Spurný et al. 1978; Hasenstein 1991; Barlow et al. 1994; Thompson and Holbrook 2004). In some cases, roots grow with scarcely perceptible lateral deflections (Erickson and Sax 1956; List 1969), whereas in others, lateral position fluctuates with both short (8 min) and long (90 min) periods (Shabala and Newman 1997a; Shabala 2003; Walter et al. 2003). In spatial analyses of maize root growth, the tip displacement velocity (of straight-growing roots) as well as elemental elongation rates throughout the growth zone fluctuate erratically (Erickson and Sax 1956; List 1969; Salamon et al. 1973). More recently, image processing methods have been applied to map the spatial profile of elemental elongation at high resolution, in maize (*Zea mays*; Walter et al. 2002, 2003) and arabidopsis (van der Weele et al. 2003). The zone of rapid elongation in maize is often bimodal, whereas several peaks are seen for arabidopsis, patterns that may indicate a regular oscillation in elongation rate as a cell traverses the growth zone.

In stems, besides the large-amplitude circumnutations discussed above, oscillations in lateral movement are often reported that may be temporally regular but are nevertheless of small amplitude. For example, species of soybean (*Glycine soja* and *G. max*) differ in whether the stem tip executes large- or small-amplitude movements but a similar period is found for each (Adolfson et al. 1998). In contrast, runner bean (*P. multiflorus*) seedling stems wander, with small-amplitude excursions (less than 5 mm) and poorly defined period (Heathcote and Idle 1965). Analysis of small-amplitude movements in the inflorescence stem of arabidopsis has shown that, although modified diurnally in period and amplitude, the movements as such are independent of the main circadian pacemaker: the diurnal modifications quickly disappear when entrained plants are moved into constant conditions (Buda et al. 2003), and arrhythmic mutants circumnutate at a constant and stable period (Dowson-Day and Millar 1999; Niinuma et al. 2005).

In contrast to the lateral movement trajectory, the linear tip velocity of stems usually fluctuates but seldom with long-lived regularity. Oscillations in displacement speed have been detected with periods of 20 to 70 min in the arabidopsis inflorescence stem (Degli Agosti et al. 1997; Jouve et al. 2000), 80 to 120 min in the azuki bean (*Vigna angularis*) stem (Gotô and Chiba 1983), and 60 and 270 min for a maize coleoptile (Liptay et al. 1995) and runner bean stems (Heathcote and Idle 1965), respectively. Oscillations so short they were termed micronutations (12- to 30-min periods) were found in runner bean tendrils, in most but not all individuals (Heathcote 1966), and even faster growth oscillations (3- to 10-min periods) occur in 1-cm-long segments of mung bean (*V. radiata*) hypocotyls (Prat and Parésys 1995; Prat et al. 1996). Both short and long oscillations in stem tip velocity, ranging from 3 to 120 min, have been observed for the seedlings of five species; surprisingly, oscillations could be detected when the position transducer was attached near the base of the hypocotyl, suggesting that elastic oscillations may also occur

(Kristie and Jolliffe 1986). In these examples, although periods are assigned, the records are noisy, and the emergence of a stable period is short lived, if it happens at all.

Erratic oscillations in stem tip velocity occur in the stems of red goosefoot (*Chenopodium rubrum*) grown under constant conditions but, under a regular photoperiod, stem velocity oscillates with a 24-h period and the erratic, higher-frequency signals vanish (Ruiz Fernandez and Wagner 1994). In contrast to the dutiful entrainment of *C. rubrum*, 3- to 4-week-old tomato (*Solanum lycopersicum*) stems grown under a photoperiod could behave more erratically: Kerckhoffs et al. (1997) recorded some (albeit not all) stems showing noisy oscillations in velocity superimposed on the regular diurnal changes.

Taken altogether, this survey suggests that highly synchronized growth oscillations, as reported for pea epicotyls (Baskin 1986) and sunflower hypocotyls (Berg and Peacock 1992), may be the exception, rather than the rule. Organs do undergo lateral movements of minor amplitude and have fluctuations in their overall extension rate, but these growth fluctuations seldom have a stable period. Admittedly, better synchrony might be visible were (elemental) growth characterized; however, given the proposed concept of facile entrainment of neighboring oscillators, one expects the entrainment to pervade the growth zone. To my knowledge, there is no example among stems or roots of a linear, ultradian growth oscillation (i.e., not out of phase on different sides of the stem) demonstrated to have the temporal stability characteristic of circumnutation in twining plants.

3.4 The Power of Bending in Plants

Pronounced, ultradian growth oscillations, although not ubiquitous, do occur and require explanation. The well-characterized growth oscillations in pea and sunflower stems take place on opposite sides of the stem, out of phase, and cause the stem to deviate appreciably from vertical. Therefore, these oscillations could be driven by gravitational overshoot. In 1973, Johnsson and Heathcote laid out the evidence pro and con for models of circumnutation based on gravitational overshoot, and concluded that gravitational overshoot was well supported. Since then, experiments in space (Brown et al. 1990) and on Earth (e.g., Hejnowicz and Sievers 1995; Obrović and Poff 1997; Yoshihara and Iino 2005) tend to suggest that circumnutation and gravitropism are separate phenomena, although able to interact.

Recently, however, support of the overshoot model was inferred by finding that circumnutation is suppressed if not eliminated in mutants of arabidopsis and morning glory (*Parbitis nil*) that lack gravitropic responsiveness in the inflorescence or main stem (Hatakeda et al. 2003; Kitazawa et al. 2005). Still, another interpretation is possible. Circumnutation in the inflorescence

stems of arabidopsis, though having a well-defined period, is small in amplitude and hence not likely to generate a significant gravitropic signal (Hatakeda et al. 2003 report wild-type amplitudes of ~200 μm). Despite lacking the ability to reorient when rotated, these mutant stems nevertheless grow vertically (the morning glory stems eventually fall over and adopt a lazy habit). One would expect random deviations (of the kind that presumably initiate an overshoot cycle) would lead the non-gravitropic stems into a wandering habit. Instead, in the absence of a gravitropic signal, it could be adaptive for the plant to suppress circumnutation. That a plant can respond by suppressing circumnutation has recently been documented for the etiolated rice coleoptile in response to red light (Yoshihara and Iino 2005), and sunflower seedlings grown in space circumnutate with diminished amplitude, and sometimes not at all (Brown et al. 1990). Conceivably, a similar response occurs in morning glory stems and arabidopsis inflorescences when gravitational responsiveness has been diminished genetically.

An alternative to oscillations based on gravitational overshoot are oscillations based on mechanical overshoot (Brown 1991; Peacock and Berg 1994). A curving stem has its convex side in compression and its concave side in tension, stresses that could in principle be sensed by the plant. And, just as the response to gravity could overshoot, so too could the response to being bent. Indeed, if an oscillating trajectory is advantageous for a growing organ, then a mechanical overshoot could be deliberate.

Remarkably, a series of experiments in favor of this idea were published over 100 years ago. Francis Darwin and Dorothea Pertz (1892) constructed a clinostat that would rotate a plant by 180° and then stop for a specific interval before making another 180° rotation. The interval between 180° rotations was usually 30 min. They used a horizontal axis of rotation to give opposite gravitropic stimuli, or a vertical axis to give opposite phototropic stimuli. The apparatus ran for many hours, and they noted the position of the stem tip every minute. Not surprisingly, this procedure set up a rhythmic bending, entrained to the alternating rotations, with phase dependent on the lag time for the gravitropic or phototropic response. But very surprisingly, after many rotations, when they deliberately failed to rotate the clinostat, the stems reversed direction anyway, just as if the apparatus had been rotated (Darwin and Pertz 1903; Fig. 3.3). In some cases, the stems reversed a second time, again just as if the alternating stimuli had continued. These results cannot be explained by gravitropic (nor phototropic) overshoot because stopping the clinostat rhythm led to the stems bending down (or away from the light); instead, it suggests that the stems were responding to the alternating mechanical flexure.

We are far more advanced in our understanding of how plants respond to light or gravity than to their mechanical status, but this status is arguably crucial to the plant. Consistent with out-of-phase growth oscillations being a response to stress in the bent region, in the circumnutating sunflower stem, growth along the entire side increases and decreases nearly synchronously (Berg and Peacock 1992; Fig. 3.2). Likewise, the peduncle of the cyclamen

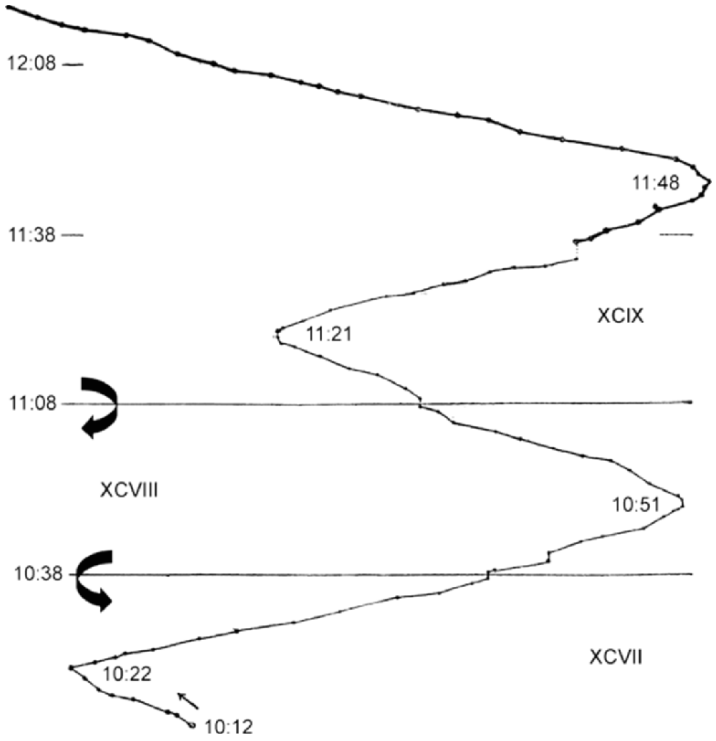

Fig. 3.3 Trajectory showing the existence of a response to bending (redrawn from Darwin and Pertz 1903). Time flows from the *bottom* to the *top*, indicated in hours:minutes by Arabic numerals. The horizontal coordinate shows the position of the stem tip in arbitrary units. A mustard (*Raphanus* sp.) seedling was placed horizontally in the custom-made clinostat, and rotated by 180° every 30 min (*thick curved arrows*). The rotation required less than 10 s, thus giving a gravitropic stimulus that changed sign every 30 min. Rotations began the day before, their total number being given by the Roman numerals. At 11:38 the clinostat was not rotated, but at 11:48 the seedling reversed direction anyway. The trajectory is drawn to show continuous movement of the plant even though the direction changed sign at each rotation. The short *vertical steps* in the trajectory at rotation times reflect the need to adjust the traveling microscope used to read the position of the stem tip. Spontaneous reversals were obtained with phototropic or gravitropic stimuli and with rotations at either 15- or 30-min intervals, and after as few as four periods. In some experiments, two reversals occurred after the clinostat stopped

(*Cyclamen hederifolium*) plant curves rapidly toward the ground as part of its dispersal mechanism, and this involves a migration of a bending growth zone at many centimeters per hour (MacDonald et al. 1987). These changes seem too rapid to reconcile with the movement of auxin, as would presumably be required for a mechanism based on gravitropism.

Responses to bending have been reported. For example, in dandelion (*Taraxacum officinale*) peduncles, a modest and transient (5 to 10 min) lateral stress elicits a vigorous growth response (Clifford et al. 1982). Recently, an ingenious series of experiments were conducted on tomato stems where the

non-growing, basal part of the stem was bent in a controlled way, and the consequent growth response in the apical part could be attributed precisely to the integrated stresses built up by the bending (Coutand and Moulia 2000; Coutand et al. 2000). To my knowledge, this is the first demonstration that plants are able to respond specifically to being bent, as opposed to a more general perturbation consequent on bending, and supports the idea that out-of-phase growth oscillations could be generated by successive responses to stem flexure.

3.5 Conclusion and Perspectives

I began with the proposition that if the growth mechanism of single plant cells within an organ is inherently oscillatory, then one expects to see those oscillations entrained and large-scale oscillations to result. This survey has shown that such oscillations in some cases are not due to growth, and in other cases are spatially and temporally erratic. From this one may suggest that either the ability to entrain the cellular oscillators is obscured by a feature of the tissue or that diffuse growth itself is not inherently oscillatory, and hence the erratic fluctuations at the organ level result from the imperfect regulation of growth among cells.

To settle this issue, measurements of relative elongation at essentially cellular resolution are crucial. Also useful would be to look for growth oscillations in single plant cells in culture that grow by diffuse growth. It might be interesting to make local perturbations, such as spot application of auxin or cellular ablation, and examine how any associated change in expansion behavior propagates through a tissue. Finally, the subject of mechanical responses requires more attention. Just as the interaction between circumnutation and gravitropism has been probed, so too the mechanical status of the organ can be manipulated and its effects on growth oscillations quantified. This endeavor would benefit from continued collaboration with engineers to develop an appropriate framework for experiments and interpretations. In this way, the power of movement in plants can eventually be understood.

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4 Nutation in Plants

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Abstract

This chapter aims to focus on the physiological aspect of oscillating growth patterns in rapidly elongating plant organs, such as roots, hypocotyls, shoots, branches and flower stalks. After a brief description of the phenomena, the theories and models proposed to date for circumnutation are reported, focusing largely on the internal oscillator model and the gravitropic overshoot model. The former is derived from the intuition of Charles Darwin, the first to suggest that circumnutatory movements are mediated by an endogenous oscillator, i.e. the driving and regulating apparatus responsible for circumnutation is internal. By contrast, the latter theory proposes a gravity-dependent model to account for circumnutations, essentially consistent with the Cholodny-Went theory, interpreting oscillations as being a continuous series of over-compensatory responses of the plant to the changing orientation of its gravisensory apparatus relative of the Earth's gravity vector. Finally, a revised two-oscillator model is reported, which is based on a combination of the above-mentioned two models. In this combined model, circumnutational movement involves a gravitropic reaction acting as an externally driven feedback oscillator, together with an endogenous or intrinsic oscillator which sends a rhythmic signal to the feedback system.

4.1 Introduction

More than a century ago, plant physiologists were already aware that rapidly elongating plant organs – roots, hypocotyls, shoots, branches, flower stalks – rarely grow in only one direction. Mean growth direction may be maintained for long intervals but the organ's instantaneous growth direction usually oscillates slowly about that mean. From a distal viewpoint, the plant organ

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Rhythms in Plants: Phenomenology, Mechanisms, and Adaptive Significance

tip, or an elongating cylindrical plant organ, describes an ellipse, a circle or pendulum-like movements about the plumbline, which can alternate between a clockwise and counter-clockwise direction. The axes of the ellipse can vary; at one extreme the ellipse approximates a line and, at the other, a circle. As the organ grows, its tip advances and (in three dimensions) traces an irregular helix. This oscillating growth pattern was well known to 19th-century plant scientists as 'revolving nutation' until the Darwins (father and son, Darwin and Darwin 1880) introduced the term 'circumnutation', used to this day (Fig. 4.1). Thus, circumnutational oscillations are manifestations of the radially asymmetric growth rate typical of elongating plant organs (Fig. 4.2). These do not include tropic processes induced by external factors such as gravity or light.

Darwin's (1875) close observation of the behaviour of 'climbing plants', of which the tendrils appeared to 'search' for some upright support, led him to widen his investigation to a large variety of species in which, however, he found no exception to his generalization that circumnutations must be a universal kind of plant movement (Darwin and Darwin 1880). Indeed, today we know that the widespread occurrence of circumnutations is even greater than Darwin had ever suspected. It occurs not only in dicots and monocots (Brown 1993) but also is well established for gymnosperms, fungi (*Basidiomycetes*), bryophytes (*Ceratodon purpureus*, Kern et al. 2005) and algae (*Spirogyra*, Kim et al. 2005). Even some colonial forms of bacteria (*Acetobacter xylinum*) exhibit oscillating growth patterns which kinematically resemble higher plant circumnutations (Hoiczyk 2000).

Although circumnutatory movements are of obvious use to twining plants seeking mechanical support, in other cases the movements appear to have no useful purpose. The amplitude, period and shape of circumnutation depend on the plant species, the plant organs involved, and the developmental stage of growth. Shoots of climbing plants (e.g. *Dioscorea batatas*, *Ipomoea quamoclit*

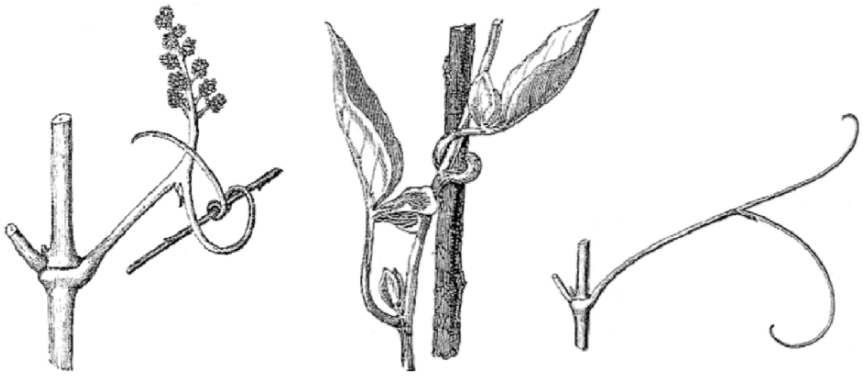

Fig. 4.1 Some sketches illustrating Darwin's close observation of the behaviour of 'climbing plants' (extracted from Darwin 1875)

Fig. 4.2 *Brassica oleracea* and circumnutation of the hypocotyl and cotyledons (extracted from Darwin and Darwin 1880)

and *Phaseolus vulgaris*) circumnutate very regularly in circular orbits (Baillaud 1962; Millet et al. 1984). By contrast, such regular circumnutation can rarely be found in more common non-climbing plants such as *Arabidopsis* (hypocotyls, Schuster and Engelmann 1997), rice (Yoshihara and Iino 2005), *Triticum* (coleoptiles, Joerrens 1959) and tulip (peduncles, Hejnowicz and Sievers 1995).

Researchers have regarded these phenomena both as oddities of plant growth and also as an outward manifestation of some important processes involved in the elongation of plant organs. Circumnutation is a growth movement, its expression depending closely on growth: whatever interferes with growth reduces or inhibits circumnutation – when tissues mature and elongation ceases, so do circumnutations. Moreover, circumnutations do not necessarily persist throughout the entire time course of organ growth. The

oscillations may be interrupted by periods of straight growth, some lasting several hours, alternating with periods of vigorous oscillations. Plant organs (shoots and roots) may oscillate either clockwise or counter-clockwise (Fig. 4.3). The same organ may stop oscillations while continuing to elongate; later, it may resume circumnutating but in the opposite direction or, without any pause, its tip may trace a figure of eight which accomplishes the reversal. Most circumnutational oscillation frequencies are in the range of 50 μHz (periods of about 20–300 min). Therefore, appropriate methods are needed to fully reveal the high incidence of circumnutational behaviour in growing plant parts. In higher plants, kinematic patterns of circumnutation are unique for each organ of a given plant. Different shoots often do not oscillate in phase and usually have different periods of oscillation.

Various mechanical stimuli can exert a dominant influence on circumnutational behaviour. Pressure (mechanical distortion), mechanical shock, subsonic vibrations, and even gentle tactile stimulation can sometimes suppress

Fig. 4.3 Clockwise and counter-clockwise oscillations of *Phaseolus vulgaris* L.

the vigour of circumnutations. It may be significant that these effects can occur within only a few minutes, often less than that needed for auxin to be transported from an organ tip to the growth region. This observation may be used as an argument in favour of a growth-control process which is local, rather than occurring in the remote tip region of the growing organ.

Beginning about 60 years ago, speculations about how plants grow and respond to tropistic stimulations were dominated by the Cholodny-Went theory (Cholodny 1926; Went 1926), according to which both the plant's environmental gravity-force detectors (statocytes) and the site of production of the growth 'hormone' are located in the apex of the responding organ. As originally proposed, the Cholodny-Went theory was chiefly concerned with the role of a chemical growth regulator in transport and its influence on the growth phases of a plant's tropistic response to a gravitational stimulus. The Cholodny-Went theory served as a guide for several generations of plant physiologists to examine and to revise. More recently, however, other naturally occurring growth regulators have been found, arguing against the 'comfortable' simplicity of views long existing in this research area. As fundamental departure from the simplified Cholodny-Went theory, evidence has been accumulating in support of a local-control theory whereby the tropistically responding region, especially of the shoot, plays a dominant role in determining the kinematics of its own response.

4.2 Theories and Models for Circumnutation

Circumnutation is the consequence of helical growth (Brown 1993) and reversible volume variations occurring in the cells of the moving part of the stem (the bending zone below the apex; Caré et al. 1998). These variations seem to be caused by the difference in water content between the convex and concave sides of the bending zone, associated with turgor and ion concentration differences between opposite sides of the stem (Fig. 4.4; Lubkin 1994). Possibly, a turgor wave rotating around the stem during circumnutation drives a helical, likely acidic growth of the stem (Hejnowicz and Sievers 1995), which we can see as stem bending. The helical growth is hypothesized to be a mechanism which increases the stability of the hypocotyls (Schuster and Engelmann 1997) during cell wall loosening (Cosgrove 2000) accompanying elongation. It has also been suggested that turgor changes are generated by endogenous, spontaneous oscillations. As a consequence, oscillatory growth and movement are generated (Van den Driessche 2000).

The cells of the bending zone communicate via plasmodesmata (Brown 1993), ion channels (Badot et al. 1990) and aquaporins (Comparot et al. 2000). Unlike pulvinary cells which are highly specialized (Engelmann 1996), no particular structure has been identified for cells in the bending zone. Circumnutations occur temporarily in young growing shoots, in the cells at a

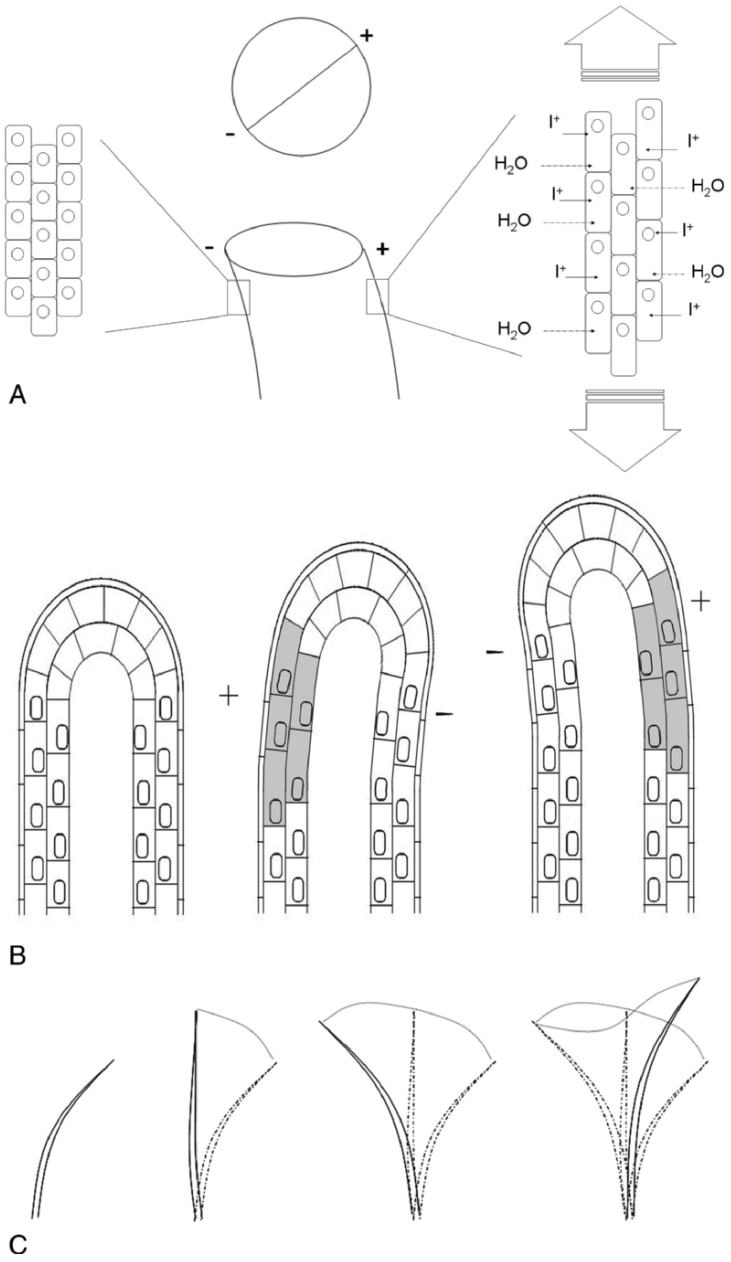

Fig. 4.4 Schematic representation of cellular turgor changes (**a**), and differential growth rate (**b**) at two opposite sides of a shoot and its effects on circumnutation (**c**)

given distance from the apex, or rather in a certain developmental stage (Van den Driessche 2000). The movements also strongly depend on light intensity, photoperiod (Buda et al. 2003), mechanical stress and temperature (Anderson-Bernadas et al. 1997).

To date, two main models for circumnutation have been proposed.

4.2.1 'Internal Oscillator' Model

Charles Darwin and his son Francis suggested that circumnutatory movements are mediated by an endogenous oscillator. Darwin tried to explain (in terms of 19th-century science) why the potential for circumnutating is ubiquitous. He considered that circumnutation is not only universal but also a fundamental process which would "be modified for the good of the plant" to accomplish tropistic or other growth responses. The Darwinian internal oscillator model is more a concept than a model, connected with the biological clock mechanism (Thain et al. 2002). Operationally, this means that the driving and regulating apparatus responsible for circumnutation is internal. Because circumnutation is patently advantageous to the plant only in a small minority of cases, researchers are not inclined to consider that it has endured only because it confers some evolutionarily significant advantage – quite the contrary – there must be something fundamental about the growth process which endows growing plant organs with the ability to circumnutate, an ability commonly displayed.

There are different hypotheses concerning the nature of an endogenous oscillator. Arnal (1953) advanced the argument that the circumnutation of coleoptiles is due to periodic variations in auxin fluxes from the tip. Moreover, Joerrens (1959) proposed that the sensitivity of the elongating cells to auxin changes periodically. Heathcote and Aston (1970) considered a hypothetical 'cellular nutational oscillator', situated in each cell and having a period equal to the periodicity of the circumnutational movement.

A recently proposed model relates to the existence of an intrinsic 'oscillator'. This model is based on the observation of strong correlations between nutation and rhythmical patterns of ion fluxes in the elongation region of corn roots (Shabala and Newman 1997; Shabala 2003). The authors noted that, when maize roots showed rhythmical movements, H^+ and Ca^{2+} fluxes also changed rhythmically, with the same average period and amplitude; when root movement was periodic, so were ion fluxes; moreover, when root growth was absent or very slow, no oscillations in ion fluxes occurred, and no nutation was observed. Shabala (2003) found that correlations between flux oscillations and root circumnutation could also be extended to include K^+. As K^+ is a major osmotic agent in plant cells and, accordingly, a main factor responsible for differential growth of root cells, an efflux of K^+ results in a loss of turgor within the cell and a consequent 'slumping' of the cell. The non-turgid cells cause asymmetric rigidity in the root, which consequently bends

to the side with less turgor (Shabala and Knowles 2002). This was further supported by direct evidence of K^+ flux oscillations closely associated with root circumnutations (Shabala 2003), the fluxes being in reversed phases when measured from opposite sides of a vertically growing root.

Circumnutation and circadian rhythms have been well studied, and there are some reports of relationships between circumnutation and biological rhythms. Schuster and Engelmann (1997) reported that arabidopsis seedlings showed a very wide range of circumnutation rhythms. In *Helianthus annuus*, circumnutation speed and trajectory length exhibit daily modulation under 16 h light/8 h dark (Buda et al. 2003). Niimura et al. (2005) demonstrated that the modulation of circumnutation speed in arabidopsis inflorescence stems is regulated by a circadian clock, pointing to the existence of an internal oscillator which regulates the speed of circumnutation. Experiments with two loss-of-function mutants, *TOC1* (mutant which shortens the period for all circadian processes analyzed to date) and *ELF3* (mutant which causes arrhythmic circadian outputs under constant white light conditions, with an almost constant nutation speed), demonstrated genetically that the circadian clock controls circumnutation speed. These results strongly confirm the hypothesis that rhythmical membrane transport processes play a key role in plant circumnutation, showing a genetic-based control.

4.2.2 'Gravitropic Overshoot' Model

When Israelsson and Johnsson (1967) proposed a gravity-dependent model to account for circumnutations, their reasoning was essentially consistent with the Cholodny-Went theory, and their theory about circumnutations proved to be an attractive explanation of how oscillations might be driven and controlled specifically by gravity. Basically, they interpreted the oscillations as being a continuous series of over-compensatory responses of the plant to the changing orientation of its gravisensory apparatus relative to the Earth's gravity vector. By interpreting the oscillations as gravity driven, their model described circumnutation as a special kind of tropistic behaviour (Fig. 4.5). The model also was consistent with the modern version of the Cholodny-Went theory for gravitropic responses, according to which both the plant's gravity detectors (statocytes) and the site of production of IAA are located in the apex of the responding organ. Nevertheless, the localization of gravisensing is much more pronounced in root tips than in shoot tips, which has to be taken into account when we try to explain circumnutations in shoots. Experiments performed under microgravity conditions aboard the Spacelab, however, revealed that gravity is not an absolute requirement either for the initiation or for the continuation of circumnutatory movements in *Helianthus annuus* hypocotyls (Brown 1993).

Kitazawa et al. (2005) demonstrated that gravisensing endodermal cells are required for shoot circumnutation in morning glory (*Pharbitis nil*). They

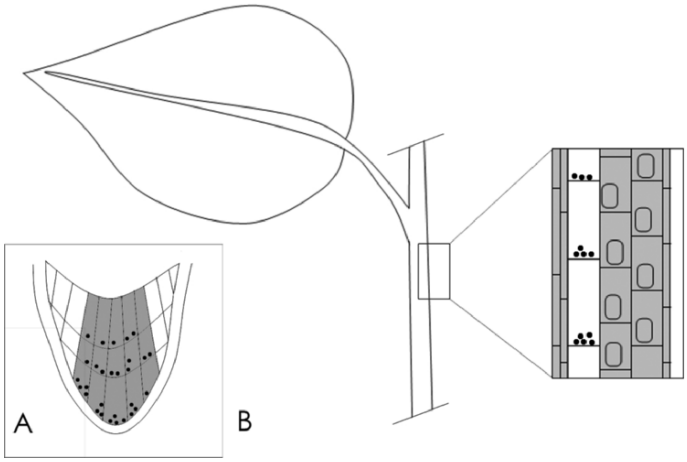

Fig. 4.5 Diagram of a root (**a**) and a stem (**b**), illustrating the regions of gravity perception

identified a gene, *PnSCR*, regulating circumnutation: the insertion of a single amino acid into the VHIID motif caused a loss of PnSCR function, resulting in an abnormal development of the endodermis required for gravisensing in the shoots of dicotyledonous plants, and suggesting that circumnutation is a gravity-dependent morphogenetic phenomenon. However, it remains obscure whether endodermis-mediated gravisensing is the sole prerequisite for circumnutation. To solve this issue, Kitazawa et al. (2005) analyzed the shoot circumnutation of two agravitropic mutants of arabidopsis, *sgr2* and *zig*/*sgr4*, which have endodermal cell layers with abnormal amyloplast sedimentation (Kato et al. 2002), finding that inflorescence stems of these mutants were defective in nutational movement. In addition, an earlier study demonstrated that circumnutation in an arabidopsis mutant, *pgm*, known to show reduced gravitropism caused by the loss of starch granules, was smaller than that of the wild type (Hatakeda et al. 2003). Together, these data corroborate the hypothesis that gravisensing and circumnutation are interlinked, demonstrating also that gravisensing cells or the endodermis-mediated graviresponse is essential for circumnutation in morning glory. The identification of *PnSCR* as the gene responsible for gravitropism in climbing plants has provided a molecular basis for elucidating the detailed mechanism of the relationship between gravisensing/graviresponse and circumnutation.

4.2.3 The ‘Mediating’ Model

Johnsson et al. (1999) proposed a revised model which combines the two models discussed above: a two-oscillator model to explain the phenomenon of circumnutation. In this model, circumnutational movement involves a

gravitropic reaction which acts as an externally driven feedback oscillator, together with an endogenous or intrinsic oscillator which sends a rhythmic signal to the feedback system. The problem remains that there has been no direct evidence yet for the involvement of the graviresponse as an external oscillator in circumnutation. Indeed, this is rather controversial, as the following discussion demonstrates.

The hypocotyls of space-flown sunflowers show circumnutation in microgravity, although the period and amplitude of the movements are relatively small (Brown 1993). Recently, Yoshihara and Iino (2005, 2006) supported the existence of a close relationship between gravitropism and circumnutation in dark-grown rice coleoptiles: (1) circumnutation was interrupted by a gravitropic response and reinitiated at a definable phase after gravitropic curvature; (2) circumnutation can be re-established by submergence and a brief gravitropic stimulation in coleoptiles which have stopped nutating in response to a red light treatment. Moreover, *lazy* mutants show no circumnutation.

Inconsistent with these results, however, Yoshihara and Iino (2006) report cases in which gravitropism and circumnutation could be separated. Firstly, the non-circumnutating *lazy* coleoptile showed nearly a wild-type level of gravitropic responsiveness in its upper half, although this part was an active site of both gravitropism and circumnutation in wild-type coleoptiles. Secondly, coleoptiles could nutate without overshooting the vertical when developing phototropic curvature. The authors concluded that gravitropism influenced, but is not directly involved in the process of circumnutation. They also suggested that a gravity signal, shared with gravitropism, contributes to the maintenance of circumnutation.

4.3 Root Circumnutation

Although there have been only occasional suggestions in earlier literature that circumnutation may aid underground organs in soil penetration (e.g. Fisher 1964), this idea has more recently gained experimental confirmation in a study on paddy rice (*Oryza sativa*) varieties (Inoue et al. 1999). Rice, an aquatic plant in origin, must have evolved to acquire traits for securing seedling establishment under flooded conditions. However, many modern varieties fail to become established under such conditions. Inoue et al. (1999) demonstrated that varietal differences in seedling-establishment percentage were attributable not to seminal root elongation rate nor apparent weight of the seed in water but rather to differences in the penetrating ability of the seminal root into soil. To examine whether root tip circumnutation could have been a facilitator of soil penetration by the root, a spectrum analysis of the root tip rotations of various varieties of rice seedlings was performed. Those seedlings which circumnutated with a frequency of 2.0–3.4 cycles per

day showed the highest seedling-establishment percentage. From these results, it appears that root tip rotations with large spiral angles are more effective in enabling the root tip to penetrate flooded or very soft soil.

In shoots, the movement has been reported to be irregular (Orbović and Poff 1997) and both right- and left-handed. In roots, by contrast, at least of the commonly studied arabidopsis ecotypes, the movement is helical and right-handed (Simmons et al. 1995). The direction towards which the arabidopsis roots slant during elongation in the wild type is considered to be the right-hand because, when the plant is viewed from above the shoot apex, the root appears to move forwards in clockwise loops – right-handed, as is known in physics. However, it should be remembered that Linnaeus and other scientists (Hashimoto 2002) considered the above movement to be left-handed because they pictured the helix from its interior, in which case the view, logically, is reversed.

In wild-type arabidopsis, root movements are not random at all but rather show a clear right-handedness, i.e. they appear to be animated by a process which could be named 'chiral circumnutation'. Mullen et al. (1998), investigating the kinetics of the gravitropic response of the *Arabidopsis* mutant *rgr1* (*r*educed root *g*ravitropism), found that the frequency of the waving pattern and circumnutation was the same in *rgr1* and in the wild type. Thus, the waving/coiling phenomenon is likely governed by circumnutation patterns. The amplitudes of these oscillations may then be selectively amplified by tactile stimulation to provide a directional preference to the slanting.

Recently, arabidopsis root movements were reinterpreted as the combined effect of essentially three processes: circumnutation, gravitropism and negative thigmotropism (Migliaccio and Piconese 2001), albeit with some difficulty in discriminating between these. Piconese et al. (2003), using an RPM (random positioning machine, which subjected the material set at its centre to a general multilateral gravistimulation, approximating space conditions), showed that the observed root pattern depended only on the circumnutating movement, since both gravitropism and negative thigmomorphism had been excluded. Using wild-type ecotypes and different gravitropic mutants (auxin transport mutants such as *aux1* and *eir1*, auxin physiology mutants such as *axr1*, handedness mutants such as *1-6C*), they observed that wild-type arabidopsis roots made large movements of circumnutation only to the right-hand but auxinic mutants, such as *aux1* and *eir1*, showed a lack of regular chiral circumnutation: auxinic mutants are disturbed not only in their gravitropic response (*aux1* and *eir1* are totally agravitropic) but also in their chiral circumnutational movement. The process destroyed in the mutants controls not only gravitropism but also circumnutation: consequently, these seem to have a common basis at the level of signal transduction (Piconese et al. 2003). Indeed, in an earlier paper, Ney and Pilet (1981) concluded that circumnutation and gravitropism had a common basis because, when the roots were responding to gravitropism, they stopped circumnutating and then resumed the movement at the end of the gravitropic response. Similar

results were obtained by the Darwins (Darwin and Darwin 1880) who, on the basis of analogous experiments, stated that gravitropism is a form of modified circumnutation, and that all plant movements have a common origin, evolved from the simple (non-chiral) movement of nutation.

The experiments reported by Piconese et al. (2003), however, limited to arabidopsis roots, cannot fully support the above hypothesis, as they show that chiral circumnutation and gravitropism in arabidopsis primary roots seem to depend on auxin transport and/or physiology. This does not imply that the processes of circumnutation and gravitropism in plants are controlled solely by auxin, which probably would be incorrect (Firn et al. 2000), but simply that this hormone seems particularly highly involved, primarily or secondarily, in the circumnutating and tropic responses of plants, as suggested from the very beginning by the pioneers of auxin research (Went and Thimann 1937).

Although several hypotheses exist as to what triggers root waving, it is clear that auxin transport and signalling are required to propagate the differential growth response once it has been triggered. Historically, auxin was thought to be transported from the shoot tip to the root, but recent evidence shows that the root tip can also synthesize auxin (Ljung et al. 2005). The asymmetric localization of auxin efflux carriers in the plasma membrane determines the polarity of transport (Galweiler et al. 1998). These carriers relocalize upon environmental stimulation and subsequently alter the overall growth response of the organ (Friml et al. 2002). Mutants of *WAV6/EIR1/AGR1/PIN2*, which encodes a putative auxin efflux facilitator, have defects in gravitropic responses and do not wave when grown on inclined hard agar plates (Okada and Shimura 1990; Luschnig et al. 1998). Mutants of *WAV5/AUX1*, which encodes a putative auxin influx carrier, are also defective in gravitropic responses but form root coils on inclined hard agar plates (Okada and Shimura 1990). Santner and Watson (2006) found that knockout mutants in the *PK3At* gene, which encodes for protein-kinase, cause aberrant growth of the primary roots of young seedlings, such that they wave. These genes were renamed *WAG1* and *WAG2*, to connote root phenotypes appearing to move to and from an agar surface.

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Part 2
Stomata Oscillations

5 Oscillations in Plant Transpiration

ANDERS JOHNSSON

Abstract

Plants take up water via the root system and transpire water vapour through stomatal openings. Surrounding *guard* and *subsidiary* cells control the magnitude of the openings, enabling transpiration but also CO_2 transport for photosynthesis. Rhythmic transpiration reflects rhythmic cellular control by these cells and shows a range of short-term periods (typically from a few minutes to over 100 min.

Hydraulic feedback models of water regulation and rhythmic transpiration via the stomatal cells have been developed, either for single or for coupled stomata oscillators. Coupling between stomata over a leaf is necessary to obtain overall transpiration rhythms. This chapter concentrates on experimental findings of transpiration rhythms and results on the occurrence of rhythms, their period, amplitude and modulation. The impact of external environmental parameters on the rhythms is dealt with, e.g. humidity, light, osmotic changes, ions.

The relevance of hydraulic feedback models is discussed as well as the possibilities of calcium oscillations in the guard cells to participate in generating the transpiration rhythms. The overall transpiration pattern can be complicated in space and time: *patchy* transpiration can occur over a leaf surface, and *period doubling* and period-n patterns have been recorded in the rhythms. There are indications that the control system can have chaotic features.

The behaviour of transpiration rhythms reveals many dynamic features of the stomatal control system. A short discussion on possible beneficial value for the plants concludes the chapter.

5.1 Introduction

Plants transpire water vapour from their leaf surfaces through *stomatal* pores. The width of the pore opening is strongly influenced by a pair of *guard* cells, and the volumes of these cells basically control the size of the pore

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opening. Measurements of water transpiration show that it can often be *rhythmic* or *oscillatory*. The pore size and its characteristics then change rhythmically, as will other variables in the water system.

The water transpiration of plants can be rhythmic under a wide variety of environmental and experimental conditions (Barrs 1971; Hopmans 1971; Cowan 1972; Raschke 1979 and others). Oscillations with a period of about one hour or less have been studied both in monocots and in dicots, whole plants and excised leaves, and they are a feature of young plants as well as of old ones. Plants showing transpiration rhythms have quite different stomatal anatomy. Furthermore, they occur in plants with varying numbers of stomata per surface area (see Kramer and Boyer 1995): about 175 per mm^2 in *Allium cepa* and about 50 per mm^2 in *Avena sativa* (in both species, on the upper and the lower leaf surface). Oscillatory transpiration is thus a general phenomenon, mirroring basic mechanisms under quite different conditions.

In a plant, the water transpired has to be replaced by water taken up from the soil/medium. It enters the root system through flow resistances and moves via the *xylem* structures into the leaves. There it reaches three dynamic elements in the water regulation of the plant: *mesophyll* cells, the *guard* cells mentioned above and the neighbouring *subsidiary* cells or *supporting tissue*. The water then evaporates through the stomatal pores. A rhythmic transpiration will be accompanied by a rhythmic water transport as well as rhythmic changes in volume, concentration of ions, membrane potentials, etc. of the cells involved. In Section 5.2, this description of water transport will be extended.

During the last decades, much interest has been focussed on oscillations in the *calcium concentration* in individual guard cells (see review by Yang et al. 2005). Ca^{2+} oscillations form a signalling pathway, and Ca^{2+} in the guard cells may act as a second messenger (McAinish et al. 1995; Yang et al. 2003 and others). These oscillations are discussed fully by McAinsh in Chapter 7 of this book. Water channels (aquaporins) might be downstream elements of Ca^{2+} oscillation signalling (Yang et al. 2005).

An overall transpiration oscillation from a leaf can be ascertained only if enough individual stomata of the leaf are *synchronized*. On the other hand, a non-rhythmic transpiration could indicate either that the individual stomata are non-oscillating *or* that they are oscillating in a *desynchronized* fashion. Individual stomata and guard cells at a short distance from each other can be desynchronized in spite of an overall oscillatory transpiration pattern (Kaiser and Kappen 2001 and others). However, a sufficiently large number of synchronized stomata must be present when rhythmic transpiration is recorded.

The synchronized, or coupled, stomata over a leaf surface constitute a *network* which is part of the control system of rhythmic water transpiration. A complete description of the oscillations should encompass features of water regulation at the molecular, ionic, cellular and physiological levels. All the different levels of the network organization are involved in rhythmic transpiration. It can thus properly be described as a "complex system" (Goldenfeld and Kadanoff 1991).

A network of cells can produce effects which are not found in studies of reactions and mechanisms in individual cells. One, therefore, has to describe the phenomena by means of appropriate models at the different levels. An effort to describe all properties of a complicated system on very basic mechanisms can be in vain. It can be too difficult a task to model a "bulldozer out from quarks" (Goldenfeld and Kadanoff 1991).

With a network concept in mind, results from measurements on oscillatory transpiration of plants are discussed in this chapter.

5.2 Models for Rhythmic Water Transpiration

5.2.1 Overall Description – "Lumped" Model

Water will be transported between two points in the plant if these have different so-called water potentials. The concept of water potential, ψ_w, is derived from the thermodynamic concept of electrochemical potential of water (see Nobel 1991). The water *flux* will be determined by the magnitude of *water potential differences*, $\Delta\psi_w$, as well as by the conductivity, g, of the medium between the two points.

Representative values of ψ_w can be found in the literature (Nobel 1991) but some examples should be given here. ψ_w in air at 95% relative humidity is about $-70 \cdot 10^5$ N/m^2 (=–70 bars), at 65% about $-700 \cdot 10^5$ N/m^2 and at 50% about $-950 \cdot 10^5$ N/m^2. Typical values for roots can be $-5 \cdot 10^5$ N/m^2, in the xylem of roots about $-6 \cdot 10^5$ N/m^2 and, in the xylem of leaves at 10-m height, $-8 \cdot 10^5$ N/m^2. All values refer to pure water under standard conditions.

Transpiration through the pores depends on the water potential difference ($\psi_{air} - \psi_{stoma}$) and the conductivity of the stomatal pore, g_s. Thus, transpiration depends on the volumes of the guard and subsidiary cells. The volumes, in turn, depend on the water content of the cells. The $\Delta\psi_w$ between ambient air and the inside of the stomatal cavity is often high, which is a prerequisite for high transpiration rates. $\psi_{air} - \psi_{stoma}$ could easily be of the order of $-900 \cdot 10^5$ N/m^2, as exemplified by the data given above.

A very simplified picture of water regulation is given in Fig. 5.1. When water is transpired from the stomatal cavity, it will be replaced from the leaf cells and from the xylem. If the water "supply" is limited, then the cells will shrink. The decrease in guard cell volume leads to a closing of the stomatal pore but the decrease in the subsidiary tissue volume tends to open the pore. The overall effect will be a closing of the pore (controlled mainly by the guard cells), *provided* that the amount of water available is restricted. On the other hand, if water "supply" is abundant, increased cell volumes lead to pore opening and closing, respectively, as shown in Fig. 5.1. The schematic figure illustrates the *feedback* principle of the water regulation.

Water supply might not always be limited. If the plant has very low root and xylem resistance to water flow, then the water supply can be looked upon

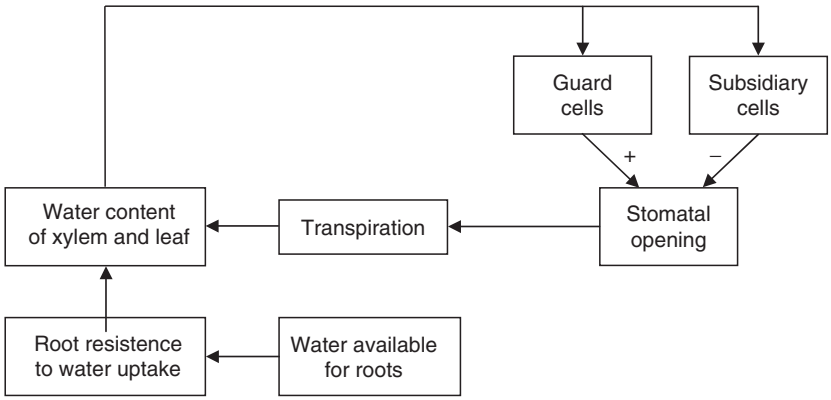

Fig. 5.1 Simplified feedback scheme for water regulation in plants. The water uptake of the plant occurs via the roots (*lowermost two boxes*). The water is transported via the xylem into the leaves (where mesophyll cells form the majority of cells). The stomatal openings in the leaf are the keys to transpiration, being affected by the guard cell volume and subsidiary cells. If the guard cell volume increases, then the stomatal opening also increases whereas it decreases when the subsidiary cells increase their volume (cf. the *positive* and *negative signs at the arrows*). The stomatal openings are decisive for plant transpiration, in turn affecting the water status of the plant. The scheme emphasizes the feedback nature of the regulatory system

as unrestricted. In this case, ψ_{xylem} does not change and the transpiration level will remain constant.

The picture outlined above is an overall description of the water transport system and forms a frame for models of overall transpiration in plants. Cowan (1972) pioneered this aspect by showing that such a system can describe an oscillatory water regulation and, thus, lead also to transpiration rhythms. It describes a *feedback control system* of the water regulation of a plant. The description forms the basis for the model used by Cowan (1972), Delwiche and Cooke (1977) and others.

It is well known, from control theory, that oscillations often arise in feedback systems under certain conditions (amplification and phase change in the loop should be large enough). A phase change in the closed loop will be particularly pronounced if there are time delays in any of the processes of the loop. Furthermore, the time constants of the volume changes are important for oscillations to arise. The guard cells and the subsidiary cells counteract each other in their effects on the stomatal pores, as illustrated by the signs in Fig. 5.1, and this can also affect the tendency to oscillate. The feedback model roughly outlined above was studied in a more elaborated form with physiological concepts, parameter values and stability criteria identified (Cowan 1972 and others).

This "lumped" model, where all the guard cells of the leaf (or the plant) have been lumped into one box denoted guard cell, all stomatal cavities lumped into one stomatal cavity, etc., explains numerous features of rhythmic transpiration. The model has also been described in mathematical

terms (e.g. Gumowski 1981, 1983). Since it contains three dynamic elements (see Sect. 5.1), its mathematical form has three state variables. It was also emphasized that any time delay in this model (in fact, any model) increases the tendency to show self-sustained oscillations. The model is nonlinear and oscillatory patterns in transpiration can, therefore, arise for suitable parameter values.

However, as we will see, there are several features of oscillatory transpiration which can not be described by this hydraulic feedback model. The pros and cons of the Cowan model were given fairly early (e.g. Johnsson 1976) but many new experimental results on the cellular level have to be taken into account in more complete models. Among these, the essentially hydraulic model has to include the questions of *how* CO_2 participates in stomatal regulation, *how* water is transported across the membranes (e.g. those of the guard cells), *how* molecular mechanisms of the guard cells affect the osmotic conditions, etc. Our information about how the water potentials are changed by cellular ion mechanisms such as Ca^{2+} oscillations and by the presence of aquaporins, abscisic acid, etc. has increased since the hydraulic models were first published. Values of flux resistances have thus to be elaborated and new concepts introduced.

5.2.2 Overall Description – "Composed" Models

Composed models have been constructed to represent a higher level of complexity and to describe how different stomata can be connected to represent a whole leaf. A short overview is given in Prytz et al. (2003a). *Coupling* between stomatal subunits can depend on the water potential in the xylem or, for instance, on a signal transmission between these. The water system can be modelled in a one-dimensional way so that *waves of rhythmic transpiration* can arise, the waves moving from subunit to subunit across a leaf surface (e.g. by adding several stomatal units to the right in Fig. 5.1).

Rand et al. (1982) found that a one-dimensional system of coupled stomatal oscillators could show spatially uniform behaviour, i.e. the different surface sections were synchronized across the leaf surface. Under non-uniform light conditions, by contrast, the system could be desynchronized and would show more complicated patterns of transpiration.

Haefner et al. (1997) coupled stomata hydraulically in a two-dimensional model. Simulations showed that all stomata oscillated synchronously but with phase differences across the leaf surface, if they were given the same parameters. However, a *patchy* organization of subunits with different amplitudes and periods emerged under conditions where stomatal parameters were spatially randomized. Thus, uncoordinated stomatal behaviour and uncoordinated transpiration occurred in this two-dimensional model.

The hydraulic coupling between different stomata (via water potential changes in the leaves) has been questioned (e.g. Kaiser and Kappen 2001) but

alternatives exist, such as electric coupling (e.g. Gradmann and Buschmann 1996), Ca^{2+} signalling (see below) or plant hormone signalling (Hetherington 2001). The next section will shortly discuss the Ca^{2+} ion and some relevant aspects.

5.2.3 Self-Sustained Guard Cell Oscillations – $(Ca^{2+})_{cyt}$ Oscillations

Cytosolic $(Ca^{2+})_{cyt}$ oscillations are known to be an information carrier in the plant signal chain, and Ca^{2+} is a signalling ion also in guard cells (see reviews by Schroeder et al. 2001, Yang et al. 2005 and others). The oscillations are detected by fluorescence techniques in individual guard cells (McAinsh et al. 1995; Trewavas and Malhó 1998 and others) and act on the single stomatal aperture. The oscillations of $(Ca^{2+})_{cyt}$ in the guard cells are treated by McAinsh in Chapter 7 of this book and only a few points will be mentioned here.

Oscillations in the $(Ca^{2+})_{cyt}$ concentration as well as calcium waves could be an endogenous basis for self-sustained volume oscillations in the guard cells (Blatt 2000; Li et al. 2004). Such $(Ca^{2+})_{cyt}$ variations can be coupled to several physiological chains in the water regulatory system, including the overt transpiration oscillations. These can influence the water channels and the transport of ions – e.g. the guard cell K^+ transport – which in turn affects the membrane voltage. The period of calcium oscillations is reported to be about 5–20 min (McAinsh et al. 1995 and others). The regularity as well as the duration of the oscillations has apparently not been studied.

In the scheme of Fig. 5.1, the $(Ca^{2+})_{cyt}$ oscillations would represent water potential changes in the guard cells. It is understood that such self-sustained oscillations in the water potential of the guard cells could produce *driven* oscillations in the hydraulic feedback model. In this way, the self-sustained guard cell oscillations could interact with possible hydraulic oscillations generated in the regulatory feedback loop. The situation can give rise to interference between two oscillating systems, as was partly explored by Prytz (2001). It was shown that complicated transpiration patterns could arise from such a system.

5.2.4 Water Channels

The membrane conductivity for water consists of one (low) conductivity path through the membrane double layers and another (high) conductivity path through water channels, also denoted aquaporins (review by Tyerman et al. 2002 and others). The water channels play an important role in plants and in the guard cell membranes, as shown in gene expression experiments (Sarda et al. 1997; Sun et al. 2001 and others; see review by Yang et al. 2005). The existence of water channels and their regulation mechanisms were not

incorporated in the feedback models discussed above but, of course, they could have been (Steudle 1997).

Experiments with $HgCl_2$ and other aquaporin inhibitors indicate that the water channels can be part of the guard cell oscillations and play "a more direct role in this process" (Yang et al. 2005). The mechanisms behind the oscillations have, however, not been mapped in detail. Cell volume changes may affect water channel activities through so-called membrane cohesion-tension (Ye et al. 2004). The water channels can also be part of the Ca^{2+} second messenger signal system and, thus, take part in self-sustained guard cell oscillations.

Lopez et al. (2004) investigated a root-specific vacuolar water channel protein which shows oscillations in the amount of transcripts. Detailed transcription studies will certainly be necessary in elucidating water channel oscillations but the presence of a root system with its water channels is not necessary for the generation of transpiration oscillations with normal period time, as described in Section 5.5.

5.2.5 Comments on Modelling Transpiration Rhythms

The hydraulic feedback models for transpiration rhythms describe parts of the complicated network system which regulates transpiration. It would be useful to review experimental results which are not easily explained by these models and also to propose critical experiments. Any model must, however, incorporate the physical parts involved in the hydraulic feedback models. The feedback models also successfully predict the outcomes of several critical experiments.

In composed models, the stomata are coupled, for instance, via the water potentials in the system and/or via calcium signalling and/or electric signalling. The coupling mechanism is important to model because the guard cells must synchronize to a certain degree in order to control rhythmic transpiration (see Sect. 5.6).

Complex modelling is needed to approach a more complete picture of the water control system in plants, which also takes into account photosynthesis and CO_2 transport through stomata (Raschke 1979). Model extensions must furthermore incorporate the network thinking as well as recent experimental findings on oscillatory cellular reactions. Only few attempts seem to have been made so far in this respect.

5.3 Basic Experimental Methods Used

Only a short summary of some methods will be given here (for further information, see textbooks on water relations of plants). Rhythmic transpiration has been recorded by enclosing the unit to be investigated (typically, a leaf or parts of it) in a container or a *cuvette* which enables a controlled air flow to

pass the unit. Modern integrated sensors can conveniently be used to record changes in humidity, CO_2, temperature, etc. of the ingoing and outgoing air.

Rhythmic water regulation often produces volume changes of stems, stalks and leaves. Measurements of transpiration oscillations and of simultaneous oscillations in other parameters of a water feedback loop provide information on the dynamical characteristics of water storage elements in the plant. Measurements of the water uptake of a plant under steady state conditions mirror the overall transpiration from the plant. The method was adopted already by Stephen Hales (1727), probably the first to publish scientifically on water relations in plants.

Stomatal conductance has been widely studied (see review by Pospisilova and Santrucek 1994) with microscopic observations of stomatal aperture (e.g. scanning EM: van Gardingen et al. 1989), vacuum infiltration of leaves by water (Beyschlag and Pfanz 1992) and porometer techniques. Gas analysis has been used to record the spatial pattern of CO_2 assimilation (Lawson and Weyers 1999). Photosynthesis assimilation rate has often been regarded as an indirect measure of the distribution of stomatal conductance and, therefore, studied by several workers (e.g. Genty and Meyer 1994). However, a simple relation between photosynthesis and conductance can be questioned (Jones 1998).

Indirect methods have also been used to study transpiration. The fact that water evaporation requires heat (about 540 cal/g water) means that transpiration through stomatal openings can cause a temperature decrease in neighbouring parts of the stomata. *Infrared thermography* has been used to map temperature rhythms of leaf surfaces simultaneously with transpiration rhythm recordings.

A method to study transpiration rhythms in excised leaves consists in replacing the root and parts of the xylem flow resistance by a physical compression of the xylem vessels (Brogårdh and Johnsson 1973). Without this increased xylem resistance, the transpiration rhythm stopped due to overflow in the plant, since ψ_{xylem} increased (see Sect. 5.2.1). The mechanically increased xylem resistance, however, decreased the water flux and, consequently, transpiration oscillations occurred. Since the roots and parts of the xylem and leaf base were removed, ions and molecules in the root medium could now enter the xylem vessels directly and eventually reach the stomatal regions (see Sect. 5.5).

5.4 Experimental Findings on Transpiration Oscillations

Transpiration rhythms are usually characterized by their period, amplitude, curve form (frequency content) and stability. Models for transpiration oscillations should be able to predict the changes in these characteristics as affected by variations in external or internal variables.

5.4.1 Occurrence of Transpiration Rhythms: Period of Rhythms

Transpiration oscillations seem to be widely found, as shown by outdoor and laboratory experiments on a variety of species. The period varies with the experimental conditions. Reports often present data for only a few cycles but, in order to meaningfully assess a value of the period, recordings of a sufficiently high number of cycles are needed. Examples can be found where the oscillations have continued for several days with variations in duration of individual cycles (see, e.g. recordings in Johnsson 1973, showing transpiration oscillations between typically 30 and 10 mm^3/h in the primary leaf of *Avena*). With these reservations in mind, the period values published for oscillations in light can be grouped into those of around a few minutes and those of around 30–60 min. Longer periods of around 100 min have been achieved in darkness or low-intensity light (Klockare et al. 1978 and others) or under the influence of certain substances (e.g. ATP, theophylline).

The rhythms can be *modulated* by a circadian rhythm; *circadian* transpiration rhythms have also been recorded (see Sect. 5.4.2.5).

5.4.2 Some Environmental Parameters Influencing Oscillations

5.4.2.1 Water Potential Conditions (Including Water Potential of the Root Medium)

The transpiration rate must be sufficiently high for oscillations to arise (g_s above zero and water potential difference $\psi_{air}-\psi_{stoma}$ sufficiently large). Under conditions of limited water supply, therefore, transpiration oscillations have often been recorded under low environmental relative humidity (e.g. Hopmans 1971; Johnsson 1973). Under conditions of water overflow of the plant (e.g. too large water access to xylem), the flow resistance must be increased to cause a subsequent opening of stomata, associated with a transpiration rhythm. Nonlinearities in stomatal conductivity play an important role for the existence and properties of rhythms (obviously, if the stomata are fully opened or fully closed, then oscillations will disappear). The period is lengthened by increased xylem resistance – as shown in experiments where the xylem has been physically compressed – and this is in agreement with the feedback models. Likewise, increased root resistance lengthens the period.

A change in the water potential of the root medium around an intact root system also changes the ψ_{xylem} and, thus, the transpiration pattern. Qualitative predictions from Cowan's model were tested for the primary leaf of *Avena*, both for continuous changes of the water potential of the root medium and for short-term treatments (Brogårdh et al. 1974). Phase shifts in the transpiration oscillations were experimentally and theoretically studied. Slight modifications in the nonlinear components of the Cowan's model were necessary to describe the results successfully. In pulse experiments, the plant

rhythm usually returns to its previous oscillatory pattern but is phase-changed. A non-oscillating state can be reached by a suitable pulse treatment (see Sect. 5.5).

5.4.2.2 Light

Most studies of transpiration rhythms have been carried out in "white" light. Studies under monochromatic light (either continuous or pulsed irradiation) remain to be performed in detail. Hopmans (1973) found in *Phaseolus* that the transpiration period was longer in broad-band blue light than in red light, and he indicated that blue light decreased the water conductivity of guard cells.

The response of guard cells and the stomatal apparatus to blue light and the chromophores mediating blue light is an active research area (see, e.g. Kinoshita et al. 2001; Paolicchi et al. 2005). Blue light photoreception has been ascribed to the carotenoid zeaxanthin and to phot1 and phot2 proteins (Kinoshita et al. 2001). Green light reversal of blue light-induced stomatal opening has been interpreted as a cycling of isomeric zeaxanthin (Talbott et al. 2002). Rhodopsin-like retinal proteins might also be involved (Paolicchi et al. 2005). The red light response is mediated via guard cell photosynthesis by chlorophyll and, thus, also depends on the CO_2 regulation of stomata. Rhythmic transpiration under blue light conditions needs to be further studied (interestingly, the phot1 protein acts on the cytosolic Ca^{2+}).

In white light, the period of the rhythm is sensitive to the irradiance level, being in general somewhat shorter at higher light intensities and longer at lower intensities (*Avena*, Klockare et al. 1978; *Phaseolus*, Hopmans 1971). When the irradiance was lowered stepwise, a major change in period occurred at about 0.2 mW/cm^2, from roughly 30 to about 110 min (Klockare et al. 1978). This longer period also occurred in darkness (see Sect. 5.7). Short light pulses can change the amplitude as well as the phase of the oscillations (*Avena*, Johnsson 1973). Both phase delays and phase advances were obtained. The transpiration oscillations could also be halted by a suitable light pulse treatment (Sect. 5.4.3).

The transpiration response to repeated light pulses showed an underlying circadian control component (period of 26–28 h). The general responses to "broad band red" and "broad band blue" light pulses were different and complex; these features will not be discussed further here (Brogårdh 1975).

5.4.2.3 Ambient Temperature and Humidity

Temperature influences transpiration rhythms: the period of the oscillations increases when temperature is lowered (about 140 min at 16 °C and about 90 min at 26 °C; Klockare and Falk 1981; see also Hopmans 1971). Sudden

changes in root temperature can induce transpiration oscillations (Hopmans 1971; Prytz 2001), which might be interpreted as a response to increased root resistance. Correspondingly, root temperature changes can induce phase shifts.

It is generally accepted that a low environmental humidity increases the tendency to transpiration oscillations, in line with the predictions discussed above (Hopmans 1971; Cowan 1972).

5.4.2.4 CO_2

Ambient CO_2 concentration influences transpiration rhythms (see, e.g. experimental results by Hopmans 1971). The hydraulic models do not include this regulation loop explicitly but do allow the water potential of the guard cell to be influenced by CO_2, much in the same way as done by light (e.g. Upadhyaya et al. 1983). Furthermore, CO_2 increases the guard cell $(Ca^{2+})_{cyt}$ (Webb et al. 1996), providing an interesting pathway to calcium oscillations.

In darkness, respiration causes high CO_2 in the stomata and one would expect these to remain closed. However, Klockare and Falk (1981) found rhythmic transpiration in darkness, the period increasing from about 40 to about 120 min when the CO_2 ambient concentration was increased from about 0.01 to about 7%. O_2 reduction to about 5% did not affect oscillations.

5.4.2.5 Modulation of Oscillations

The parameters in the water control system change in a daily manner: root resistance, mean level of g_s, etc. – all vary throughout the day (Barrs and Klepper 1968; Hopmans 1971; Cowan 1972). These changes might then modulate the short-term transpiration oscillations. Their amplitudes are reported to increase towards the end of the day but usually decrease to essentially zero at night. These responses are all likely to be controlled in a *circadian* manner, i.e. regulated by a biological clock with a period close to but different from 24 h. Willmer and Fricker (1996) give some examples showing circadian transpiration measurements, and Brinker et al. (2001) confirm circadian stomatal movements in Gymnosperm species. Also *Arabidopsis* shows circadian transpiration (Fig. 2 in Webb 1998).

The transpiration response to light steps has been shown to be modulated in a circadian fashion (the period being about 27 h; this modulation was ascribed to circadian CO_2 variations in leaves; Brogårdh and Johnsson 1975a).

The fact that light pulses change the phase and amplitude of the rhythms enables studies of the resonance of the transpiration system. This possibility has partly been explored by using sinusoidal white light signals to entrain the oscillations in different parts of the primary *Avena* leaf (Brogårdh and

Johnsson 1974b). The entrainment range was not investigated and this approach might be worth pursuing further.

5.4.3 Singularities of Transpiration Rhythms: Test of Models

A short-term *perturbation* of an oscillating system often results in a temporary deviation from its regular path. When the perturbation ceases, the rhythm might resume its former shape but then often showing changes in *phase* and *amplitude*.

Originally discussed for circadian rhythms, the question has been asked if biological oscillators in general could reach so-called *singularities* or *fixed points*. An oscillator in such a state is characterized by a halt in all oscillating variables – it has *stopped oscillating* (temporarily or permanently). This state might be obtained by perturbations affecting the amplitude of the rhythm, e.g. light pulses. Winfree (1970) explored the possibilities of stopping a circadian rhythm in *Drosophila* by administering blue light pulses of suitable irradiance and duration at a certain phase of the rhythm. His results could be interpreted as if the rhythm had indeed been stopped due to a precisely administered light pulse of correct amplitude.

Along this line of thought, white light pulse experiments on the *Avena* transpiration rhythm demonstrated, firstly, the phase resetting ability of light and dark pulses and the amplitude effects, much as in Winfree's experiments (Johnsson 1973). Secondly, it was shown that a suitable pulse or combination of pulses could, in fact, halt the transpiration rhythm (Fig. 5.2). The oscillatory

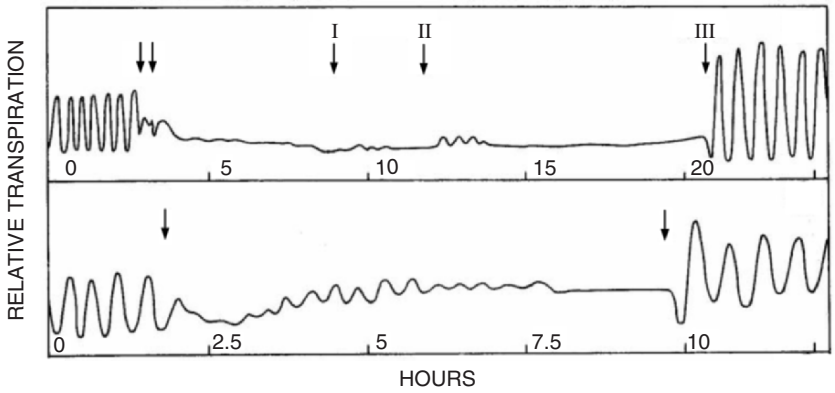

Fig. 5.2 Light/dark perturbations stopping transpiration rhythms in *Avena* plants. In the *upper recording*, a combination of two pulses stopped the transpiration rhythm at about 4 h. *Arrows I*, *II* and *III* indicate white light pulses with duration of 30 s, 2 min and 10 min respectively. The two first pulses cause small oscillatory patterns but only a sufficiently strong pulse, III, can start the rhythm anew. In the *lower recording*, the dampened transpiration rhythm spontaneously shows an oscillatory tendency after about 5 h but eventually returns to a standstill. A stronger light pulse starts the oscillations again (redrawn after Johnsson et al. 1979, with permission)

system was not irreversibly damaged, since a subsequent strong light pulse could initiate the rhythm again. The experiments were interpreted to demonstrate that a singularity had been achieved (Johnsson et al. 1979).

The nature of this particular state seemed to be stable in some cases; in others, it was evidently unstable because the halted rhythm could spontaneously resume oscillating (see Fig. 5.2).

The transpiration oscillations could also be stopped transiently by *osmotic* pulses (–32 bars, 3 or 6 min; Johnsson et al. 1979). This indicates that the action of the light perturbations in the singularity experiments might be mediated via osmotic perturbations of the water control system. Although these results were not possible to simulate with Cowan's model as published, special nonlinear features introduced into the nonlinearity between the transpiration rate and the stomatal conductance enabled successful simulations. Singularity experiments could be critical tests of models for transpiration rhythms.

5.5 Ionic Interference with Transpiration Oscillations

Intact plant roots represent an efficient barrier towards many ions and molecules. For the study of ion influences on rhythmic transpiration and stomata, the xylem compression method (Sect. 5.3) has been used. The method allows transpiration oscillations to continue and, simultaneously, ions to enter the stomatal regions (Brogårdh and Johnsson 1975b). In some cases, an effect on the transpiration rhythm could be detected already in the first oscillatory cycle after onset of administration of the ions (effects on period, amplitude or curve form of transpiration rhythm).

Ca^{2+} (20 mM chloride salt), Mg^{2+} (40 mM) and La^{3+} (2.5 mM) given to the xylem as *pulses* all prolonged the period of the transpiration oscillations, with a concurrent increase in the amplitude of the rhythms (Brogårdh and Johnsson 1975b). Effects were reversible at the concentrations used. This period lengthening was ascribed either to ionic effects in the guard cells, e.g. affecting the K^+ transports, or to changes in water permeability of the guard cells. Simulations using the Cowan models with decreased water permeability of the guard cell membranes caused the same type of changes as those found experimentally.

Lithium chloride salt – given permanently to intact plants – caused no period changes (plants had a period of about 40 min). Applying a pulse of 80 mM LiCl to the cut end of leaves, however, led to a rapid increase (about 10%) in the period of the oscillation – within the first cycle after onset of the pulse (Brogårdh and Johnsson 1974a). Li^+ caused a period increase in a dose-dependent manner, and a 25-min *pulse* of 40 mM LiCl caused a reversible period lengthening.

At the time, the action of the lithium ions was interpreted to occur preferentially via ion pumping mechanisms but, today, alternative interpretations

are obvious. The ion has been shown to act on glycogen synthase kinase-3 as well as on processes involving inositol metabolism with several relevant downstream targets (see Quiroz et al. 2004). These reaction chains can be relevant in the case of the stomatal control system. Lithium slows down circadian rhythms in general, acts on aquaporins in renal functions, etc. and has a broad spectrum of action mechanisms.

These lithium effects could be due to interference with calcium oscillations and calcium signalling pathways in guard cells. The experiments should, therefore, be repeated in studies on guard cell oscillations, to see if corresponding period and amplitude changes are experimentally found.

The short discussion given above deals with experiments of fairly short duration, mostly of some hours only. The same is true of experiments in which 0.1% theophylline, kinetin, ATP or valinomycin has been administered to oscillating *Avena* leaves. In several cases, the substances cause dramatic period lengthening (Johnsson 1976). Other ions (e.g. H^+, Cl^-) as well as abscisic acid, etc. are known to be involved in guard cell reactions (see, e.g. Yang et al. 2005) but the direct action on rhythmic transpiration remains to be studied in detail.

5.6 Patchy Water Transpiration from Leaf Surface

In most studies, leaf surfaces have been treated as a whole and bulk models might, in such cases, be useful to describe rhythmic transpiration. By contrast, the *coupling* and *interaction* between stomata have remained relatively unexplored (Mott and Buckley 2000).

In several cases, *infrared studies of the temperature variations* of the leaf surface have been performed. Kümmerlen et al. (1999) found a linear relationship between transpiration rate and leaf temperature change. Infrared imaging, or thermography, has been used to estimate stomatal conductance both in the field (see recent paper by Cohen et al. 2005) and in the laboratory. This enables investigations of the spatial and temporal variations of stomatal conductance across leaf surfaces. Jones (1999) showed that the technique has sufficient spatial resolution to yield information on the variability of stomatal conductance across the surface of *Phaseolus vulgaris* leaves.

Evidence exists of a *desynchronization* of stomata across the leaf surface, whereby some areas, or *patches*, behave differently than others (Pospisilova and Santrucek 1994; Mott and Buckley 2000 and others). Mott et al. (1999) emphasized the importance of hydraulic interactions among stomata. They hypothesized and presented experimental evidence that the hydraulic coupling between stomata depended on evaporation and, therefore, on ambient humidity. Patchy stomatal conductance, and its relation to the so-called carbon–water balance, was quantitatively discussed by Buckley et al. (1999) and others. Siebke and Weis (1995) observed that bulk gas exchange values

can remain constant despite different areas of the leaf oscillating non-synchronously and patchily.

Prytz et al. (2003a) investigated the temporal and spatial variations in oscillatory transpiration across the surface of primary oat leaves by means of detailed thermography. The overall transpiration from the leaf was recorded simultaneously. During rhythmic behaviour, the entire leaf surface displayed, by and large, the same temporal leaf temperature pattern as that recorded for whole-leaf transpiration (although patch-like temperature variations did occur sometimes). Small phase differences across the leaf surface (distal regions lagging 0.5–3 min behind the central leaf region) were observed in the rhythms. This synchronous behaviour during oscillatory transpiration indicates strong coupling between stomata in the primary *Avena* leaf (low ambient relative humidity, cf. Mott et al. 1999).

5.7 Period Doubling and Bifurcations in Transpiration – a Way to Chaos?

Several "lumped" models for the water regulatory system use three dynamic variables or elements (Sect. 5.2.1). Coupled models have a correspondingly higher number of dynamic variables. A nonlinear model with three variables can show *period doubling* and *chaotic behaviour* (Strogatz 1994). Complicated, non-sinusoidal waveforms have been encountered experimentally (Johnsson 1976; Johnsson and Prytz 2002) and some efforts to model their shape have been published (Gumowski 1983). If time delays are introduced into the reactions, then the overall model becomes even more apt to show oscillations.

An example of so-called *period doubling* in the water transpiration of the primary *Avena* leaf is given in Fig. 5.3 (upper curve). One can conveniently introduce the concept of *period-n oscillation* (Strogatz 1994), characterized by a pattern repeating itself every *n*th maximum in transpiration. A period doubling is thus a period-2 oscillatory behaviour.

Period doubling is but one of the many complicated oscillatory patterns demonstrated in transpiration rhythms. The term *bifurcation* is used in the mathematical literature, and the phenomenon can be found in many nonlinear model systems at a critical value of the system's control or bifurcation parameter. Period doubling was found in simulations of a mathematical model of the water regulatory system (for a description, see Johnsson and Prytz 2002), essentially based on the Cowan model. Further changes in the control parameter result in successive bifurcations giving period-4, period-8, period-16 oscillations, and so on. After an infinite number of bifurcations, the system becomes *aperiodic* or *chaotic*, and the period doubling phenomenon is often an indication of a system approaching such a chaotic state. In the chaotic region period-3, period-5, period-6 oscillations and others may exist.

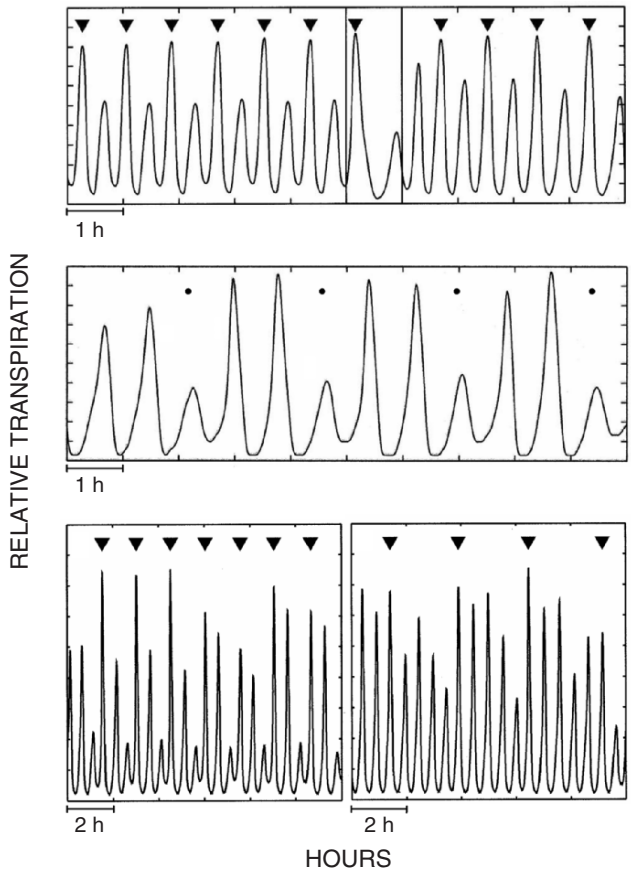

Fig. 5.3 Complex oscillatory transpiration patterns. *Upper curve* Period-2 oscillations, the *triangles* indicating maxima in the rhythm (every second peak). A phase shift is induced by a pulse perturbation of 80 mM KCl to standard medium (*centre*). *Middle curve* Recording showing period-3 oscillations in an intact plant, the *circles* indicating minima in the rhythm (every third peak distinctly smaller than the rest). *Lower curve*, *left* Period-3 oscillations, the *triangles* indicating maxima at every third peak (20 mM KCl added to medium). The pattern differs from that of the period-3 oscillations in the middle curve. *Right* Recording of a plant showing period-5 oscillations, the *triangles* indicating maxima (20 mM KCl added to root medium; redrawn after Prytz et al. 2003b, with permission)

Few studies have been performed to search for period doubling sequences of the water control system. One attempt was made by Klockare et al. (1978) who varied light intensity (see Sect. 5.4.2.2). A rather abrupt transition in period, from about 30 to 110 min (see Sect. 5.4.2.2), was encountered when irradiance was lower than about 0.2 mW/cm^2. At the transition level, the transpiration rhythms showed irregular curve shapes and autocorrelation analysis indicated that both the periods recorded could occur simultaneously.

Prytz et al. (2003b) demonstrated that the primary *Avena* leaf can show rather complicated rhythms which are sometimes very complex (examples in Fig. 5.3). Thus, the overall transpiration system approaches (under certain conditions) chaotic behaviour. Period doubling sequences have been reported in physical and chemical as well as biological systems (see Cvitanovic 1989; Lloyd 1997 and references therein). However, studies of such oscillating systems are, to my knowledge, very rare in plant physiology (Shabala et al. 1997).

These transpiration oscillations were recorded when the potassium concentration in the xylem medium was increased and used as a control parameter. As is well known, the K^+ ion plays a crucial role in guard cell dynamics. It would therefore be highly interesting to investigate possible simultaneous bifurcation patterns in transpiration rhythms and in calcium concentration and calcium chain-controlled rhythms.

5.8 Conclusions

This chapter has focussed on direct measurements of transpiration rhythms under different physicochemical conditions. The literature shows that oscillatory transpiration can exist in the water regulatory feedback system (hydraulic feedback system). At the same time, basic cellular reactions have been shown to oscillate in guard cells. It is, therefore, of interest to study the interaction between $(Ca^{2+})_{cyt}$ reactions and the hydraulic system. Not only calcium oscillations but also abscisic acid signalling and the presence of aquaporins are central within this context. Experiments should be designed to study $(Ca^{2+})_{cyt}$ oscillations and self-sustained transpiration rhythms simultaneously in order to complete and improve existing models.

It seems to be of basic interest to determine whether or not Ca^{2+} oscillations always show the same period as that of the transpiration rhythms, and whether or not these possess singularities. Do they show complex curve forms, as found for transpiration (cf. Sects. 5.4 to 5.7)? Only experiments can demonstrate the existence and nature of the possible coupling between transpiration rhythms and $(Ca^{2+})_{cyt}$ oscillations.

Novel findings on this topic will undoubtedly improve our models of the oscillatory transpiration of plants. Incorporating the gating of water transport into models (with relevant classes of aquaporins; see Tyerman et al. 2002) as well as results from experiments focussing on the nature of the coupling between stomatal regions will further increase the precision of models. Finally, spectral studies of transpiration and Ca^{2+} oscillations should be performed simultaneously. The possible approach to chaos under varying conditions is another task to be tackled.

It is likely that models have to be developed at a network level (see Sect. 5.1). Necessarily, they will mirror our knowledge on different levels: the

hydraulic feedback level as well as the detailed cellular and molecular levels. The time constants involved in the oscillatory patterns (longer for transpiration oscillations, shorter for calcium oscillations?) should be matched in suitable ways (see, e.g. Lloyd and Lloyd 1995).

As is the case for many short-term oscillatory processes in biology, one can formulate a basic question: why oscillate at all? The answers are still tentative but bear on important physiological and evolutionary aspects.

A rapid control system (and rapid responses might be favourable in the stomatal control) has tendencies to show "unwanted" oscillations. To keep a stable control (and, in our case, a balanced and constant water loss) is usually demanding in a control system and it has, therefore, been argued that transpiration oscillations could represent overshoots in the control system.

However, another approach to a fundamental answer is taken by considering the relationship between water transpiration and the CO_2 absorption of leaves (e.g. Cowan 1977). A decrease in stomatal pore size reduces water transpiration as well as CO_2 absorption but water transport reduction is proportionally more affected. The so-called WUE (water use efficiency) therefore varies as a function of stomatal conductivity. Now, it is recalled that WUE can, in fact, increase during limited water supply – if oscillatory behaviour is present. This points to the beneficial nature of these oscillations (Upadhyaya 1988 and others; see review by Yang et al. 2005). However, it seems reasonable to state that further studies are recommended to substantiate the data currently available on this aspect.

It is also worth pointing out that information on biological *chaotic* systems can be relevant. Chaotic systems (as found, e.g. in the heart rhythm) can use their sensitivity to stabilize a control by means of small perturbations, thereby increasing their flexibility and speed in response to varying environmental conditions (see, e.g. Shinbrot et al. 1993; Lloyd and Lloyd 1995 and others). Such systems might be of major biological value in several contexts (Lloyd and Lloyd 1995) and can be of advantage also for the regulation of water transpiration. Chaotic systems are also flexible mechanisms for rhythmic behaviour (Strogatz 1994; Lloyd and Lloyd 1995).

It is not an easy task to establish whether a biological system is chaotic by nature, especially not from time series with limited amounts of data and with limited numbers of oscillatory periods. The complex oscillatory patterns of the transpiration rhythms discussed in Section 5.7 might, however, be of interest within this context, pointing to a possible chaotic underlying system.

The oscillatory transpiration of plants reveals much of the dynamics of an important biological control system. As has been emphasized by Cowan (1977), the stomatal system provides – more directly than any other aspects of the functions of plants – a way to understand the dilemma of land plants, viz. to accumulate carbon without excessive loss of water. Detailed experimental studies of these oscillations represent a key approach to increasing our knowledge of some of the most basic processes in plants.

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6 Membrane Transport and Ca^{2+} Oscillations in Guard Cells

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Abstract

Since the 1980s, work on ion transport and the control of guard cell ion channels has provided a wealth of information that is still unparalleled in plant biology, driven primarily by electrophysiological studies and, more recently, by molecular genetics and cell biology. We know now sufficient detail of all of the major transport pathways at the plasma membrane to encapsulate these fully with accurate kinetics and flux equations in which all of the key parameters are constrained by experimental data. Both experimental and modelling (so-called systems biology) approaches have already yielded important insights into oscillatory signal interactions, especially in relation to Ca^{2+} and Ca^{2+}-dependent signal processing. Critical to understanding these events is a recognition of the capacity for feedback that is inherent to ion transport across a single membrane, and embodied in the common intermediates of ion concentrations and membrane voltage. Here, we review this background and its relevance to Ca^{2+} signals and oscillations that have been demonstrated to occur in guard cells, and we place this evidence in context to support that short-term oscillations in solute transport are the norm for homeostatic control of osmotic content.

6.1 Introduction

Guard cells surround pores (stomata) within the epidermis of all aerial parts of most plants. The guard cells open the stoma to permit gas exchange and CO_2 entry, and they close the stoma to prevent water vapour loss from the intercellular spaces within the plant tissues to the environment. From a practical standpoint, guard cells thus affect two processes most important to the vegetative plant, namely photosynthesis and transpiration. So, an understanding of signal processing that controls stomatal movements leads directly to more

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applied aspects including vegetative yield, water conservation and agricultural management (Shinozaki and Yamaguchi-Shinosaki 1999; Schroeder et al. 2001b; Hetherington and Woodward 2003; Gedney et al. 2006). Because the demands for CO_2 in photosynthesis and for water retention are frequently at odds, guard cells must integrate these as well as other signals to give fine balance between the open and closed states of the stoma. The quest to understand how guard cells achieve this balance has fuelled research on this 'model' plant cell. Since the 1980s, work on ion transport and the control of guard cell ion channels has provided a wealth of information that is still unparalleled in plant biology, driven primarily by electrophysiological studies and, more recently, by molecular genetics and cell biology. We know now sufficient detail of all of the major transport pathways at the plasma membrane to encapsulate these fully with accurate kinetics and flux equations in which all of the key parameters are constrained by experimental data. Indeed, integrative (so-called systems biology) approaches have already demonstrated the capacity for describing known physiological behaviours and predicting new ones, notably in relation to oscillatory signal interactions with membrane ion transport (Gradmann et al. 1993; Blatt 2000).

This chapter reviews the background to oscillations in guard cell signalling, the experimental evidence and their cellular mechanisms, and it explores some of the most salient issues that face our understanding of guard cells. We give special attention to oscillations in Ca^{2+} and Ca^{2+}-dependent signalling, as these are particularly relevant to understanding the fine-tuning of solute fluxes that drive stomatal movements, and they have broad relevance to signal processing in plants generally.

6.2 Oscillations and the Membrane Platform

Oscillatory behaviour is a common feature of many biological systems. It has a special place in signal transduction for which the balance between two quasi-stable (or 'bi-stable') states often performs the function of a 'switch', either enabling or disabling signal transmission. Good examples of such 'switching' functions are found in the binding/debinding and nucleotide hydrolysis characteristics of small GTPases (Yang 2002; Vernoud et al. 2003) and in protein (de-)phosphorylation mediated by protein kinase C (Violin et al. 2003). Oscillatory behaviour is also a feature of signal cascade 'poise' – its characteristic for rapid and definitive response – for the simple reason that it reflects an inherent instability and thereby contributes a sensitivity to inputs that may easily drive (or bias) the switch in one direction. These characteristics, too, are well-established elements of biological signalling events, including those dependent on Ca^{2+} (Tsien and Tsien 1990; Blatt 2000; Hepler et al. 2001; Violin et al. 2003). The reader will find excellent discussion of the concepts in the broader context of bifurcation theory and synthetic biology in

Elowitz et al. (2002), Chin (2006), and in the reviews of Paulsson and Ehrenberg (2001) and Kaern et al. (2003).

Underlying all oscillatory phenomena are two or more interacting elements that are coupled and feed back on one another. While feedback is a common feature of regulatory networks – in biochemical pathways, often mediated through allosteric control of a late product on an earlier enzymatic step and separate from the biosynthetic pathway (Segel 1993) [what we may term *extrinsic kinetic feedback*] – it is uniquely inherent to transport across membranes. Unlike any other biological network, membrane transport engages enzymatic reactions that operate in antiparallel fashion across the membrane bilayer, at the same time sharing common substrates and products (Blatt 2004).

Why should the membrane offer such a unique platform for oscillatory interaction? From an enzymologist's viewpoint, membrane transporters are proteins that facilitate the conversion of substrates to products, thereby consuming or releasing free energy. It happens only that these changes in free energy relate to the electrochemical states of the substrates and products on the two sides of the membrane, rather than to any chemical bonding (the chemical identities of the transported ions and compounds are the same, whether the transport mechanism entails coupling to H^+ or passive diffusion through an ion channel). Indeed, membrane physiologists make use of conventional reaction-kinetic paradigms, incorporating ion and other substrate concentrations, cyclic binding and debinding steps, to give the same mathematical substance to transport as with any other enzyme kinetic process. The energetic input for transport across all biological membranes is achieved by coupling ATP hydrolysis to the transport of a 'driver ion' to generate a thermodynamic gradient for this ion across the membrane (or the reverse in the case of mitochondria and chloroplasts, which work in reverse by using the thermodynamically 'downhill' flux of the H^+ to power ATP synthesis). In turn, dissipation of this driver-ion gradient is then coupled through other discrete transport proteins in order to energise the transport of a wide range of solutes, both organic and inorganic. For example, the plant plasma membrane is energised by H^+-ATPases that hydrolyse ATP in order to pump H^+ out of the cell and generate a thermodynamic gradient of H^+ ($\Delta\mu_H$) directed back into the cell across the membrane. In turn, a number of transporters tap into this gradient, coupling the downhill flux of H^+ back into the cell to the movement of other solutes. Examples in this case include H^+-coupled sugar and amino-acid symporters (Slayman and Slayman 1974; Schwab and Komor 1978), H^+-coupled NO_3^- transport (Meharg and Blatt 1995) and Na^+ export through H^+-coupled Na^+ antiporters (Clint and MacRobbie 1987; Shi et al. 2000).

Significantly, the association across a common membrane of these two subsets of transporters – those that generate the driver-ion gradient and those that dissipate it – leads to a situation characterised by what we may consider as *intrinsic kinetic feedback* in which the substrate for one process is the product of the other, and vice versa (see Fig. 6.1). It is at the heart of

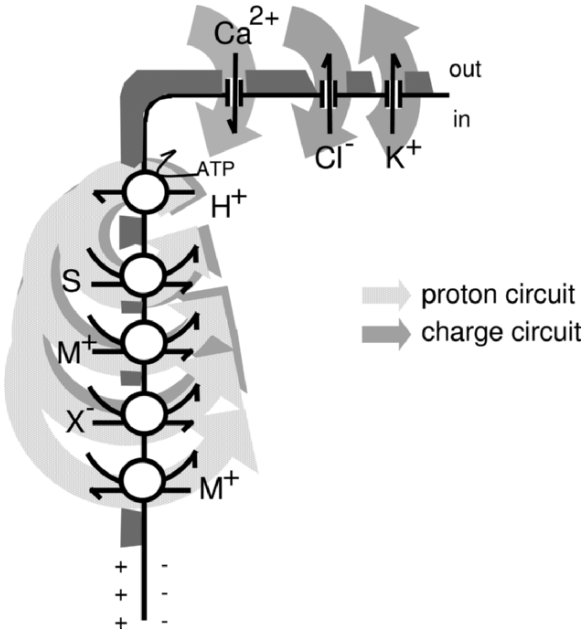

Fig. 6.1 Schematic of proton and charge circuits at the plant plasma membrane. ATP hydrolysis drives H^+ out of the cell, generating both charge and H^+ gradients ($\Delta\mu_H$) to energise transport. Coupled transport with many charged (M^+, X^-) and uncharged (S) solutes contributes to the return pathways of the H^+ and electrical circuits, examples including coupled uptake of NO_3^- with two H^+ (Meharg and Blatt 1995; Blatt et al. 1997); ion channels (Ca^{2+}, K^+, Cl^-) contribute only to the electrical circuit

Mitchell's chemiosmotic hypothesis (Mitchell 1969) and comprises the H^+ circuit of the membrane, a (nearly) closed pathway of two parts, one generating and the other dissipating the H^+ gradient. Through this circuit, H^+ ions cycle in and out across the membrane, at once both substrate and product on one side of the membrane, and product and substrate on the other. Thus, at the most fundamental level, biological membranes and transport across them comprise a platform for oscillatory behaviour.

Membrane voltage adds one more facet to these intrinsic interactions across biological membranes. Because the vast majority of these transporters carry electrical charge associated with the transported solute(s), one consequence of transport is that it affects the distribution of charge across the membrane and, hence, the membrane voltage. By the same token, charges that move across a membrane necessarily do so through an electric field (at the macroscopic level, the membrane voltage) and, therefore, will be affected both in rate and direction by changes in membrane voltage. The essence of these statements is obvious but their significance is less often appreciated: membrane voltage affects the kinetic characteristics of transport in much the

same way as does ion (substrate or product) concentration on either side of the membrane. In a very real sense, membrane voltage is both an electrical 'substrate' and 'product' for all charge-carrying transporters. In other words, membrane voltage is a common medium, even more so than the H^+, and is shared between all charge-carrying transporters, including ion channels (see Fig. 6.1).

6.3 Elements of Guard Cell Ion Transport

The very large ion fluxes associated with stomatal movements offer a particularly useful handle for analysis of transport control in stomatal guard cells. Between open and closed states, the guard cells of *Vicia*, for example, take up or release 2–4 pmol of KCl. On a cell volume basis, these changes correspond to 200–300 mOsM in solute content. Since mature guard cells lack plasmodesmata (Wille and Lucas 1984), all of this solute flux must pass across the plasma membrane. The plasma membrane of guard cells, similarly to that of plant cells generally, is energised by H^+-ATPases that drive H^+ out of the cell, generating an electrochemical gradient for H^+ directed inwards across the membrane. Analyses of the *Vicia* guard cell H^+-ATPase has shown that one H^+ is transported for each molecule of ATP hydrolysed (Blatt 1987). This activity maintains a gradient of 2–3 pH units and a membrane voltage of −150 to −200 mV (inside negative) under most conditions. At near-neutral pH and with typical concentrations of ATP, ADP and inorganic phosphate in the cytosol, the maximum equivalent output of the H^+-ATPase predicts a stalling voltage near −500 mV, a point at which no net ATP hydrolysis nor H^+ transport occurs. The fact that the membrane voltage rarely exceeds −300 mV, even when the external pH is near 7, is to be expected since the pump is used to do work in generating the $\Delta\mu_H$ to move other solutes across the membrane. Again, the circuits of current (charge flux) and H^+ pass through the pump and return via these other transport mechanisms. In fact, electrophysiological analyses have confirmed that the guard cell H^+-ATPase is more than sufficient to account for solute uptake during stomatal opening (Blatt 1987; Lohse and Hedrich 1992). There is evidence that *Vicia* guard cells express unusually high levels of two distinct H^+-ATPases at the plasma membrane (Villalba et al. 1991; Becker et al. 1993; Hentzen et al. 1996), consistent with the higher demand expected for membrane energisation in the guard cells. Otherwise, H^+ extrusion is regulated in common with other plant H^+-ATPases, including control by cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and 14-3-3-mediated phosphorylation (Goh et al. 1995; Kinoshita and Shimazaki 1999; Shimazaki et al. 1999).

Little is known still about H^+-coupled transport in guard cells, although it is clear that coupled uptake of K^+ (Blatt and Clint 1989; Clint and Blatt 1989) and of anions, especially Cl^- (MacRobbie 1981, 1984), must occur. By contrast,

we have a wealth of knowledge about the ion channels of guard cells, both at the plasma membrane and, to a lesser extent, at the tonoplast. Comparisons of these channels between species, in those instances for which comparable data are available, indicate some (albeit subtle) differences in kinetic and/or regulatory characteristics. However, the general patterns are largely the same (Blatt 2000; Hetherington 2001; Schroeder et al. 2001a; Very and Sentenac 2003; Dreyer et al. 2004). The plasma membrane is dominated by three major channel currents. Under most circumstances, two classes of K^+ channels are prevalent that are separable on the basis of their biophysical and pharmacological properties as well as their molecular identities. Current through inward-rectifying K^+ channels ($I_{K,in}$) facilitates K^+ uptake during stomatal opening and, in guard cells of *Arabidopsis*, is identified principally with current through the KAT1 K^+ channel (Nakamura et al. 1995; Blatt 2000; Hetherington 2001; Very and Sentenac 2003; Dreyer et al. 2004; but see also Pilot et al. 2001). KAT1 and related K^+ currents in planta show a requirement for millimolar $[K^+]$ outside (Blatt 1992; Thiel et al. 1992; Hertel et al. 2005) but gating is otherwise essentially independent of $[K^+]$ (Schroeder 1988; Blatt 1992). The guard cell $I_{K,in}$ is strongly suppressed by cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$), in *Vicia* guard cells showing an apparent K_i around 300 nM and a high (fourfold) degree of cooperativity but only a minor sensitivity to cytosolic pH, pH_i (Grabov and Blatt 1997, 1999). Current carried by outward-rectifying K^+ channels ($I_{K,out}$) provides the major pathway for K^+ efflux during stomatal closure (Clint and Blatt 1989; Blatt 2000; Schroeder et al. 2001a) and, in *Arabidopsis*, is identified exclusively with the GORK K^+ channel (Hosy et al. 2003). Again, a common set of physiological and biophysical characteristics have been observed between species, notably an unusual voltage sensitivity that coordinates with $[K^+]$ outside (Blatt 1988; Blatt and Gradmann 1997; Roelfsema and Prins 1997) and is intrinsic to this subfamily of K^+ channels (Gaymard et al. 1998; Hosy et al. 2003; Johansson et al. 2006). By contrast with $I_{K,in}$, activation of $I_{K,out}$ is unaffected by $[Ca^{2+}]_i$ (Hosoi et al. 1988) but is strongly dependent on pH_i (Blatt 1992; Blatt and Armstrong 1993; Miedema and Assmann 1996; Grabov and Blatt 1997).

The third class of channels at the guard cell plasma membrane carry Cl^- as well as other anions. Anion efflux is important for stomatal closure to bring the membrane voltage positive of E_K for net loss of K^+ through $I_{K,out}$. Two Cl^- (anion) channels have been identified with different macroscopic current kinetics (Hedrich et al. 1990; Linder and Raschke 1992; Schroeder and Keller 1992), and may constitute different functional 'modes' of the same channel protein (Dietrich and Hedrich 1994; Thomine et al. 1995). Of these, one current (S-type (Schroeder and Keller 1992) or SLAC (Linder and Raschke 1992) current, hereafter identified as I_{Cl}) activates and deactivates slowly (halftimes, 5–30 s) on voltage steps, and exhibits a significant conductance at voltages between −150 and −200 mV, characteristics that are consistent with the prolonged (20–30 min) ion flux that occurs during stomatal closure (Grabov et al. 1997; Pei et al. 1997; Roelfsema et al. 2004).

By contrast with events at the plasma membrane, our understanding of transport across the tonoplast is relatively poor. As in virtually all mature plant cells, the vacuole in guard cells makes up 80–90% of the total cell volume. Thus, the greater proportion of solutes that pass across the plasma membrane during stomatal movements must also traverse the tonoplast, and this in a coordinated manner (MacRobbie 1995, 1999). Furthermore, the vacuole is an important source and sink for Ca^{2+} and H^+, and therefore is expected to contribute to signalling events that lead to changes in the free concentration of these ions in the cytosol (Frohnmeyer et al. 1998; Leckie et al. 1998). In common with other plant vacuolar membranes, the guard cell tonoplast harbours at least two different cation channels that are capable of carrying K^+ and Ca^{2+} (Ward and Schroeder 1994; Schulzlessdorf and Hedrich 1995; Tikhonova et al. 1997), and an anion channel with high selectivity for Cl^- over K^+ and dependent on protein phosphorylation for activity (Pei et al. 1996). Of these, the so-called slow-vacuolar (SV) channel was recently identified with the Ca^{2+}-permeant TPC1 channel protein (Peiter et al. 2005), and the vacuolar-K^+ (VK) channel has been suggested to correspond with the TPK1 (=KCO1) cation channel (Bihler et al. 2005). Both of these cation channels are likely to be important for solute loss and stomatal closure but their respective contributions still remain to be explored in detail.

6.4 Ca^{2+} and Voltage

How is a coordinate regulation of these several ion transporters achieved? Consider, for a moment, the three channel populations at the guard cell plasma membrane. Clearly, all three channels, and the H^+-ATPase, locate within the same membrane and, therefore, interact through the common 'intermediate' of the membrane voltage. So, it is no surprise that the voltage sensitivities of both K^+ channels and the Cl^- channel currents at the plasma membrane contribute significantly to their regulation under free-running (that is, in non-voltage-clamp) conditions (Gradmann et al. 1993). All three channels are subject to a second tier of controls as well, and these account for the ability of stomata to integrate a wide range of hormonal and environmental factors (Willmer and Fricker 1996). During stomatal closure triggered by the water-stress hormone abscisic acid (ABA), for example, signalling inputs that regulate the K^+ and Cl^- channels include G-proteins and sphingolipids (Ng et al. 2001; Wang et al. 2001; Coursol et al. 2005), inositol phosphates (Lee et al. 1996; Lemtiri-Chlieh et al. 2000; Hunt et al. 2003), protein (de-)phosphorylation (Armstrong et al. 1995; Li et al. 2000; Mustilli et al. 2002; Assmann 2003), reactive oxygen species (Kwak et al. 2003; Desikan et al. 2005), nitric oxide (Garcia-Mata et al. 2003; Sokolovski et al. 2005) and pH_i (Irving et al. 1992; Blatt and Armstrong 1993; Grabov and Blatt 1997). These signals notwithstanding, a recurring theme centres around changes in $[Ca^{2+}]_i$.

Significantly, $[Ca^{2+}]_i$ signalling, and its associated oscillations, brings us directly back to events at the plasma membrane and membrane voltage. Thus, we can now reconstruct major control elements and their downstream responses in a functional 'signalling cassette'.

6.4.1 The Ca^{2+} Theme

Resting $[Ca^{2+}]_i$ in guard cells, as in other eukaryotic cells, is situated near 100 nM but may be elevated to micromolar free concentrations (Gilroy et al. 1990; McAinsh et al. 1990; Grabov and Blatt 1997, 1999; Allen et al. 2001). Above resting $[Ca^{2+}]_i$, small increases in free divalent reduce $I_{K,in}$ with an apparent K_i of 300 nM and a cooperativity coefficient of 4 such that, at 500 nM $[Ca^{2+}]_i$, the K^+ channels are essentially inactive (Grabov and Blatt 1999). Detailed analysis of the kinetics has shown that increasing $[Ca^{2+}]_i$ displaces the voltage sensitivity of K^+ channel gating out of the normal physiological voltage range (approx. −50 to −200 mV), so that $I_{K,in}$ is not active under free-running (non-voltage-clamp) conditions (Grabov and Blatt 1997, 1999). Increasing $[Ca^{2+}]_i$ also promotes I_{Cl} (Hedrich et al. 1990; Schroeder and Keller 1992), although quantitative kinetic detail is still incomplete. Significantly, ABA itself evokes qualitatively similar changes in both $I_{K,in}$ and I_{Cl} (Thiel et al. 1992; Lemtiri-Chlieh and MacRobbie 1994; Grabov et al. 1997; Pei et al. 1997) that have been correlated with $[Ca^{2+}]_i$ increases, in some cases to values above 1 μM (Fricker et al. 1991; Irving et al. 1992; McAinsh et al. 1992; Allan et al. 1994). However, even a relatively small rise in $[Ca^{2+}]_i$ is clearly sufficient to suppress $I_{K,in}$ (Grabov and Blatt 1999) and probably to promote I_{Cl}, thereby depolarising the membrane to bias it for solute efflux.

Elevation of $[Ca^{2+}]_i$ in guard cells depends both on Ca^{2+} entry across the plasma membrane and on release from intracellular stores (Blatt 2000; Hetherington 2001). In response to ABA, Ca^{2+} entry from outside appears to be mediated by a single population of Ca^{2+} channels in the plasma membrane that are kinetically distinct from K^+ channels, show a high selectivity for Ca^{2+} and Ba^{2+} and, by contrast with mammalian Ca^{2+} channels, are activated at negative membrane voltages (Hamilton et al. 2000, 2001). Hamilton et al. (2000) found this Ca^{2+} current (I_{Ca}) to be potentiated by ABA and strongly suppressed by micromolar $[Ca^{2+}]_i$, indicating a self-regulating feedback mechanism. Intriguingly, I_{Ca} was promoted by ABA even in excised membrane patches, an observation that may indicate a regulatory protein complex associated with the channels and, thus, relate to subsequent observations that both protein phosphorylation (Köhler and Blatt 2002) and NADPH oxidases (Kwak et al. 2003) affect Ca^{2+} channel activity.

By contrast, intracellular Ca^{2+} release in guard cells appears to proceed via at least three different pathways, including stores that are subject to activation by inositol-1,4,5-trisphosphate (Blatt et al. 1990; Gilroy et al. 1990; Parmar and Brearley 1995; Lee et al. 1996), by inositol hexakisphosphate

(Lemtiri-Chlieh et al. 2000), and by cyclic ADP-ribose (cADPR), a metabolite of nictoinamide adenine dinucleotide (Grabov and Blatt 1998; Leckie et al. 1998). Of these, the cADPR-activated (ryanodine-sensitive) Ca^{2+}-release channels are evidently of particular importance. Leckie et al. (1998) observed $[Ca^{2+}]_i$ to rise following injections of guard cells with cADPR, and they noted that stomatal closure in ABA was slowed when guard cells were preloaded with the cADPR antagonist 8-NH_2-cADPR. Grabov and Blatt (1998, 1999) and Hamilton et al. (2000) found that the ABA-evoked $[Ca^{2+}]_i$ signals were sensitive to ryanodine and that ABA strongly influenced the kinetics for the $[Ca^{2+}]_i$ rise and its recovery, indicating that ABA must act both on Ca^{2+} entry across the plasma membrane and on its release from cADPR-dependent Ca^{2+} stores.

The discoveries that nitric oxide (NO) promotes drought tolerance and that NO scavengers suppress stomatal closure (Garcia-Mata and Lamattina 2001, 2002; Neill et al. 2002) has provided another piece to the puzzle of $[Ca^{2+}]_i$-mediated signalling in guard cells. Garcia-Mata et al. (2003) added essential molecular detail, showing that low nanomolar levels (<10 nM) of NO elevated $[Ca^{2+}]_i$, and were essential for normal inactivation of $I_{K,in}$ and activation of I_{Cl} in ABA. The $[Ca^{2+}]_i$ rise evoked by NO was sensitive to the antagonist of guanylate cyclase ODQ (1-H-(1, 2, 4)-oxadiazole-[4,3-a] quinolxalin-1-one) and to ryanodine, consistent with a signal cascade analogous to the canonical pathways mediated via cGMP, cADPR and ryanodine-sensitive Ca^{2+} channels in animals (Stamler and Meissner 2001; Lamattina et al. 2003). Subsequent work demonstrated that NO-mediated Ca^{2+} release is modulated by protein phosphorylation (Sokolovski et al. 2005). The juxtaposition of these observations and evidence for cGMP as a downstream component in the NO-induced $[Ca^{2+}]_i$ rise does suggest an additional role for the cGMP-dependent protein kinase G, although direct evidence is yet forthcoming. This caveat aside, then, the molecular mechanics of $[Ca^{2+}]_i$ elevation follows a well-defined pattern: (1) Ca^{2+} enters across the plasma membrane through voltage-gated Ca^{2+} channels that are activated by a change in membrane voltage, and (2) Ca^{2+} entry triggers Ca^{2+} release from intracellular stores via endomembrane channels, the activity of which additionally (3) is modulated by elevated cADPR through an NO cascade of NO synthase, guanylate cyclase and cGMP-stimulated adenylate cyclase. Nonetheless, one important aspect distinguishes this Ca^{2+} signal cascade from that of the animal models and has a direct bearing on $[Ca^{2+}]_i$ oscillations: in the guard cells, membrane hyperpolarisation is required to activate the plasma membrane Ca^{2+} channels (Hamilton et al. 2000).

6.4.2 $[Ca^{2+}]_i$ Oscillations

The question how many different developmental and homeostatic responses might be controlled by $[Ca^{2+}]_i$, even in one cell type, was resolved only when it was recognised that repetitive increases in $[Ca^{2+}]_i$ might contain information

about the nature of the signal in its frequency and location within the cell (Meyer and Stryer 1991; Berridge 1998; Neher 1998). These oscillations arise when a local $[Ca^{2+}]_i$ rise triggers further Ca^{2+} release from intracellular stores (so-called Ca^{2+}-induced Ca^{2+} release or CICR) before the Ca^{2+} is eliminated from the cell or sequestered within organelles. The frequency of $[Ca^{2+}]_i$ increases or 'spikes' has now been shown to encode 'Ca^{2+} signatures' specific for expression of selected genes (Dolmetsch et al. 1997, 1998) and calmodulin-dependent protein kinase activity (DeKoninck and Schulman 1998).

Frequency encoding of Ca^{2+} signals is equally common in plants – for example, in response to external stimuli (Ehrhardt et al. 1996; Bauer et al. 1997, 1998; Engstrom et al. 2002) and during polar development (Holdaway-Clarke et al. 1998; Coelho et al. 2002; Holdaway-Clarke and Hepler 2003). In guard cells, $[Ca^{2+}]_i$ oscillations were first identified with changes in CO_2 and the extracellular ionic environment (McAinsh et al. 1995; Webb et al. 1996) and, subsequently, with ABA and oxidative stress (Staxen et al. 1999; Allen et al. 2000). However, these oscillations occurred generally with periods of 10 min or more, and with discrete $[Ca^{2+}]_i$ maxima lasting 1–4 min. In other words, the changes in $[Ca^{2+}]_i$ were so slow, compared with the time course for stomatal closure, that they could not encode directly for the closing stimulus. For example, Staxen et al. (1999) reported $[Ca^{2+}]_i$ oscillations that began within the first 15–30 min of ABA exposures and were maintained with a period of approximately 8–10 min over 1–2 h; yet, closure itself under these conditions would have been achieved before the first $[Ca^{2+}]_i$ cycle was completed (Willmer and Fricker 1996). So, the significance of $[Ca^{2+}]_i$ oscillations in guard cells remains something of a puzzle.

One clue to a role for $[Ca^{2+}]_i$ oscillations in this case may rest with the finding that their entrainment appears to determine the subsequent recovery of the guard cells and stomatal opening. Allen et al. (2001) found that closure occurred rapidly when $[Ca^{2+}]_i$ was elevated under experimental control, regardless of oscillation period or duration, but that the degree of long-term steady-state closure depended on $[Ca^{2+}]_i$ oscillations within a defined range of frequency, transient number, duration and amplitude. These results suggest an oscillation-associated 'memory' that may play an adaptive role in stomatal behaviour; indeed, it is a long-standing observation that a history of drought or ABA treatment protects plants from subsequent water stress (Wright 1969; Wright and Hiron 1969). Nonetheless, this interpretation largely ignores another, direct relationship between the $[Ca^{2+}]_i$ oscillations and ionic homeostasis in the guard cells.

6.4.3 Voltage Oscillations

Guard cells, like the cells of many plants, show two states of the membrane. One state is characterised by voltages close to the K^+ equilibrium voltage (E_K) and by conductances that are mediated by $I_{K,out}$ and I_{Cl}; the second state is

typified by voltages well-negative of E_K and shows a conductance that is mediated by the H^+-ATPase and, to a lesser extent, by $I_{K,in}$ (Thiel et al. 1992; Gradmann et al. 1993). Thus, in principle, these two states reflect the bias of the membrane, in the first case for K^+ and Cl^- loss and, in the second, for their accumulation within the guard cell (see Fig. 2 in Blatt 1991 for graphic current-voltage analysis). Significantly, oscillations between these states occur in response to stimuli, including ABA, with time periods often comparable to those of the oscillations in $[Ca^{2+}]_i$ (Thiel et al. 1992; Blatt and Armstrong 1993; Blatt and Thiel 1994). Gradmann et al. (1993) applied a systems biology approach based on quantitative kinetic modelling to show that oscillations with periods on the order of 10 s could be maintained with a minimum of the three ion channel currents ($I_{K,in}$, $I_{K,out}$, I_{Cl}) and the H^+-ATPase under physiologically meaningful constraints (an energy-dependent Cl^- uptake mechanism was included for completeness). There are some important limitations to this model. For example, it included 'activation' and 'inactivation' characteristics for the H^+-ATPase and Cl^- uptake as simplifying ('black box') assumptions to accommodate kinetic relaxations in response to changes in voltage. Furthermore, the model did not include contributions from I_{Ca}, although it was noted that additional factors such as mediated by $[Ca^{2+}]_i$ might be incorporated to add further control for the oscillations. Nonetheless, the model showed the capacity to reproduce voltage oscillations and conductance characteristics of experimental records. More still, it predicted a homeostatic balance of osmotic solute content that could be shifted between net K^+ and Cl^- uptake and their loss, with very minor changes to the kinetic parameters for the individual currents. Gradmann et al. (1993) concluded that osmotic balance "is accomplished, not by a steady-state but by transitions between two stable states, one of salt uptake at voltages considerably more negative than E_K, and another one of salt release at voltages some 10 mV more positive than E_K."

6.4.4 Membrane Voltage and the '$[Ca^{2+}]_i$ Cassette'

Work from this laboratory first yielded the link between $[Ca^{2+}]_i$ and membrane voltage, providing a key element of the framework to understanding the molecular mechanics behind their oscillations. Grabov and Blatt (1998) observed that spontaneous oscillations in free-running (non-clamped) membrane voltage were accompanied by oscillations in $[Ca^{2+}]_i$: when the voltage hyperpolarised, this was closely followed by a rise in $[Ca^{2+}]_i$, which recovered whenever the voltage depolarised. The same pattern was recovered under voltage clamp when the membrane was driven experimentally between −200 and −50 mV, and when the voltage was manipulated by changing extracellular $[K^+]$. Measurements with different $[Ca^{2+}]$ outside showed that the $[Ca^{2+}]_i$ rise evoked by voltage was dependent on Ca^{2+} entry across the plasma membrane, and analysis of its voltage-dependence showed a sharp threshold

between −110 and −140 mV with a mean near −120 mV. Furthermore, this threshold was shifted, on average, by more than +40 mV to values near −70 mV in the presence of ABA. These findings pointed directly to a Ca^{2+} channel that was gated by negative membrane voltage, and led to subsequent discoveries of hyperpolarisation-activated Ca^{2+} channels at the plasma membranes of guard cells (Hamilton et al. 2000) and in other plant cells (Very and Davies 2000; Kiegle et al. 2000). It also formed the basis for experimentally imposed $[Ca^{2+}]_i$ oscillations and the idea of a 'oscillation memory' in stomatal movements (Allen et al. 2001).

More important still, the observations of voltage-dependent $[Ca^{2+}]_i$ increases yielded direct evidence of what we have termed a $[Ca^{2+}]_i$ 'signalling cassette' to describe its role as part of a feedback mechanism controlling the balance between the two states of the membrane (Blatt 2000). A synopsis of this signalling cassette comprises four steps (see Fig. 6.2): (1) with resting $[Ca^{2+}]_i$ low, negative membrane voltage was seen to trigger Ca^{2+} influx across the plasma membrane, which stimulated intracellular Ca^{2+} release to elevate

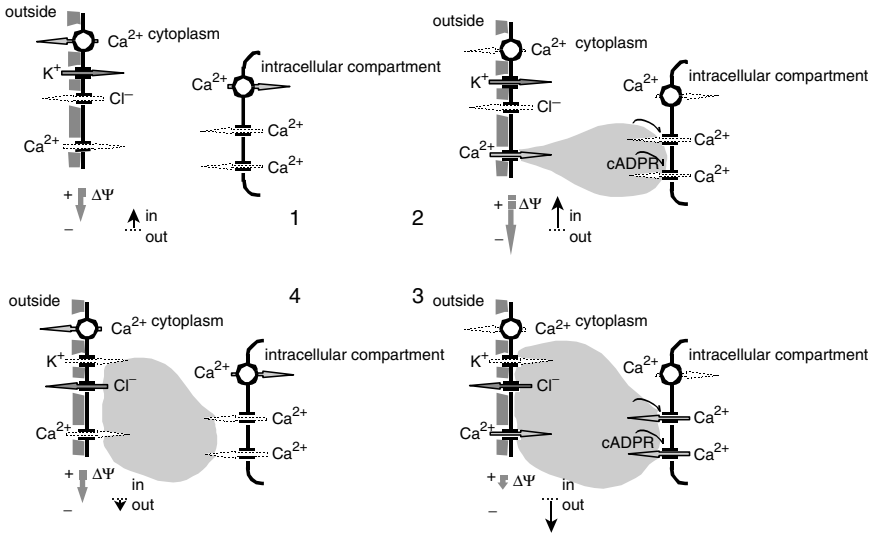

Fig. 6.2 The voltage–$[Ca^{2+}]_i$ response 'cassette' cycle, clockwise from top left. *Horizontal arrows* indicate ion fluxes (*dashed* inactive, *shaded* active). *Arrows* below each frame indicate sign and amplitude of membrane voltage ($\Delta\Psi$, *shaded arrow*), net K^+ and Cl^- fluxes (*arrow above/below dotted line*, *in*/*out*). Negative membrane voltage triggers Ca^{2+} influx across the plasma membrane (frames *1* and *2*), which stimulates intracellular Ca^{2+} release to raise $[Ca^{2+}]_i$ (frame *3*, local $[Ca^{2+}]_i$ indicated by *shading*), activate I_{Cl} and inactivate $I_{K,in}$. Cl^- efflux and additional changes in membrane conductance (not indicated) drive membrane depolarisation. Membrane depolarisation and the elevated $[Ca^{2+}]_i$ inactivate the Ca^{2+} influx and engage Ca^{2+} pumps (frames *3* and *4*). With $[Ca^{2+}]_i$ reduced, I_{Cl} inactivates and the membrane hyperpolarises to reactivate $I_{K,in}$ (frame *1*)

$[Ca^{2+}]_i$; (2) the rise in $[Ca^{2+}]_i$, in turn, inactivated $I_{K,in}$ as well as the Ca^{2+} influx, and activated the Ca^{2+}-sensitive Cl^- current I_{Cl}, promoting membrane depolarisation; (3) depolarisation favoured K^+ and Cl^- efflux and, with the Ca^{2+} influx suppressed, permitted Ca^{2+} pumps to re-sequester Ca^{2+} and lower $[Ca^{2+}]_i$; finally, (4) with the fall in $[Ca^{2+}]_i$, I_{Cl} reduced sufficiently for the membrane to repolarise and drive K^+ uptake through the reactivated $I_{K,in}$. More simply put, the process is a 'ping-pong' cycle of controls exerted by $[Ca^{2+}]_i$ and voltage, with negative voltage driving a rise in $[Ca^{2+}]_i$, elevated $[Ca^{2+}]_i$ driving depolarisation, depolarisation facilitating $[Ca^{2+}]_i$ recovery, and $[Ca^{2+}]_i$ recovery driving membrane hyperpolarisation; at the core of this process is the intrinsic feedback of membrane transporters operating across a common membrane, as we noted at the beginning of this chapter. What is the significance of this $[Ca^{2+}]_i$ signalling cassette?

Clearly, the voltage threshold for Ca^{2+} entry and $[Ca^{2+}]_i$ increases is strongly affected by external signals such as ABA (Grabov and Blatt 1998; Hamilton et al. 2000), with the consequent bias to the second half of the cycle described above – in other words, favouring $[Ca^{2+}]_i$ elevation, membrane depolarisation. These observations are entirely in harmony with the predictions of quantitative modelling and the final outcome of a shift to K^+ and Cl^- efflux (Gradmann et al. 1993). In short, we see this interplay of membrane voltage and $[Ca^{2+}]_i$ oscillations as a homeostatic mechanism that 'tunes' the guard cell plasma membrane to achieve different positions in a spectrum of time-averaged balance points between osmotic solute uptake and its loss.

6.5 Concluding Remarks

It is clear now that a very large number of signalling elements and related factors contribute to the regulation of guard cell transport. Naturally, some of the key questions centre around the implied mechanisms, the relative importance to stomatal function in each case and, therefore, the nature and degree of interactions between these signalling elements. Establishing hierarchies often poses a major difficulty, especially in relation to Ca^{2+} channels and $[Ca^{2+}]_i$ signalling for which cyclic or oscillatory behaviours are a prevalent feature. These, in turn, have attracted considerable attention with concepts of frequency encoding that give biological 'substance' to the oscillations themselves but, perhaps, at the expense of understanding the significance of their origins. A close look at ion transport across membranes shows a commonality of biophysical and kinetic properties inherent to transport that are at the very core of coupled oscillations in biology. For guard cells, it also shows that osmotic solute transport is an integral part of the oscillatory behaviour associated with $[Ca^{2+}]_i$ signalling – what we previously described as a $[Ca^{2+}]_i$ 'signalling cassette'. Quantitative ('systems') modelling supports this interpretation, predicting that short-term oscillations in solute transport are the

norm for homeostatic control of osmotic content. It will be interesting to see, now, whether these predictions are validated and the insights that this may yield.

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7 Calcium Oscillations in Guard Cell Adaptive Responses to the Environment

MARTIN R. MCAINSH

Abstract

Ca^{2+} has been shown to be a ubiquitous intracellular second messenger in plant cells, raising the question of how specificity is controlled in Ca^{2+}-based signalling systems in plants. There is considerable interest in the possibility that stimulus-induced oscillations in the cytosolic concentration of free Ca^{2+} can encode information used to specify the outcome of the final response through the generation of stimulus-specific Ca^{2+} signatures. Recent results provide good evidence that, at least in stomatal guard cells, signalling information is encoded in oscillations and transients in $[Ca^{2+}]_{cyt}$, enabling stomata to formulate the optimal stomatal aperture to balance CO_2 uptake for photosynthesis and water loss through transpiration under a specific set of environmental conditions. These findings are discussed here, together with models for the encryption and decoding of the signalling information encoded in guard cell Ca^{2+} signatures.

7.1 Introduction

Twenty years ago, the role of the calcium ion (Ca^{2+}) as a signalling molecule in plants was far from understood and researchers in this field faced significant conceptual and technical challenges, many of which were novel to plant systems, when trying to elucidate the importance of Ca^{2+}-based signalling systems in plants (Fig. 7.1). Today, Ca^{2+} is firmly established as a ubiquitous intracellular second messenger in plant cells. An increase in the cytosolic concentration of free Ca^{2+} ($[Ca^{2+}]_{cyt}$) has been shown to be an important component in the signal transduction pathways by which a diverse array of environmental and developmental stimuli are coupled to their respective end response (Rudd and Franklin-Tong 2001; Sanders et al. 2002; White and Broadley 2003; Hetherington and Brownlee 2004). However, the very ubiquity of this second messenger has prompted researchers to question how specificity

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Fig. 7.1 Advice from a caterpillar

is controlled in Ca^{2+}-based signalling systems (McAinsh and Hetherington 1998; Evans et al. 2001; Ng and McAinsh 2003).

Work on mammalian cells has indicated that information about the type and severity of a stimulus can be encrypted in the spatiotemporal characteristics of stimulus-induced changes in $[Ca^{2+}]_{cyt}$ (for reviews, see Fewtrell 1993; Berridge et al. 2003). It is clear that plants have a similar capacity to generate complex spatiotemporal patterns of $[Ca^{2+}]_{cyt}$ changes, including transients, spikes and oscillations (for reviews, see Rudd and Franklin-Tong 2001; Evans et al. 2001; Ng and McAinsh 2003), and it has been suggested that these also have the potential to encode stimulus-specific signalling information in plant cells through the generation of unique Ca^{2+} signatures (McAinsh and

Hetherington 1998; Harper 2001; Rudd and Franklin-Tong 2001; Evans et al. 2001; Schroeder et al. 2001; Sanders et al. 2002; Ng and McAinsh 2003; Hetherington and Brownlee 2004).

The mechanisms by which Ca^{2+} signals are generated in plant cells involve the coordinated action of plasma membrane and endomembrane Ca^{2+}-permeable channels, Ca^{2+}-ATPases and H^+/Ca^{2+} exchangers, and Ca^{2+}-binding proteins. These have been extensively reviewed by White (2000), Sanders et al. (2002) and Hetherington and Brownlee (2004), and are treated elsewhere in this book (see Chap. 6 by Blatt et al.). Therefore, this chapter will consider only the physiological significance of oscillations in plant $[Ca^{2+}]_{cyt}$, focusing specifically on the stomatal guard cell.

7.2 Guard Cells and Specificity in Ca^{2+} Signalling

Stimulus-induced oscillations in $[Ca^{2+}]_{cyt}$ have been observed in a number of different plant cells (for a review, see Evans et al. 2001), including pollen tubes (Holdaway-Clark and Hepler 2003), root hairs (Shaw and Long 2003) and stomatal guard cells (Fan et al. 2004). Of these, it is the extensive body of data on stomatal guard cells that provides the most compelling evidence for an important physiological role of $[Ca^{2+}]_{cyt}$ oscillations in plant Ca^{2+} signalling. So, why have guard cells been used so successfully to study the role of $[Ca^{2+}]_{cyt}$ and oscillations in $[Ca^{2+}]_{cyt}$ in plant signalling pathways?

The answer to this question lies, at least in part, in the fact that guard cells respond to a wide range of environmental stimuli in an easily quantifiable manner (a change in cell turgor, associated with a corresponding change in stomatal aperture) and that they are also tractable to many of the cell physiological and molecular techniques required for the measurement and manipulation of $[Ca^{2+}]_{cyt}$ dynamics. In addition to these more technical considerations, the regulation of gas exchange by guard cells provides a clear illustration of the physiological importance of specificity in plant Ca^{2+} signalling. Guard cells must integrate signals from a range of often conflicting stimuli such as light, elevated $[CO_2]$, temperature and plant hormones, many of which have been shown to induce a change in guard cell $[Ca^{2+}]_{cyt}$ (Sanders et al. 2002; White and Broadley 2003; Hetherington and Brownlee 2004) when formulating the optimal stomatal aperture to balance CO_2 uptake for photosynthesis and water loss through transpiration under a specific set of environmental conditions. For example, guard cells must contain the signalling machinery required to enable them to distinguish between the plant hormones auxin (Irving et al. 1992) and abscisic acid, ABA (McAinsh et al. 1990), which have opposite effects on stomatal aperture, i.e. stimulating opening and closing respectively, but which both induce an increase in guard cell $[Ca^{2+}]_{cyt}$. They also must contain the signalling machinery necessary to produce a graded, rather than an 'all or nothing' response to different magnitudes of a given Ca^{2+}-mobilizing stimulus such as ABA (McAinsh et al. 1991).

Consequently, although our understanding of how complex spatiotemporal patterns of $[Ca^{2+}]_{cyt}$ changes contribute to specificity in plant Ca^{2+} signalling is still in its infancy – it has even been argued recently that Ca^{2+} may often act as a switch in plant signalling pathways and that specificity is encoded elsewhere in the signalling pathway (Scrase-Field and Knight 2003) – there is good evidence that, at least in guard cells, signalling information is encoded in oscillations and transients in $[Ca^{2+}]_{cyt}$.

7.3 Ca^{2+} Signatures: Encoding Specificity in Ca^{2+} Signals

Studies in animal cells suggest that fluctuations in both the spatial and temporal dynamics of stimulus-induced changes in $[Ca^{2+}]_{cyt}$ contribute to the generation of Ca^{2+} signatures, and spatial heterogeneities and localized increases in $[Ca^{2+}]_{cyt}$ are known to play an important role in the encryption of stimulus-specific signalling information (for reviews, see Fewtrell 1993; Berridge et al. 2003). For example, it has been shown that elevations in nuclear Ca^{2+} in cells of the AtT20 mouse pituitary cell line control Ca^{2+}-activated gene expression via the cyclic AMP response element, while increases in $[Ca^{2+}]_{cyt}$ regulate gene expression through the serum response element (Hardingham et al. 1997). Similarly, in embryos of the brown alga *Fucus*, the observation of variations in the spatiotemporal dynamics of hypo-osmotic shock-induced Ca^{2+} waves has led Goddard et al. (2000) to propose that differences in the signature of the Ca^{2+} wave determine downstream physiological responses such that specific patterns of Ca^{2+} wave generation encode the necessary information for differential regulation of cell volume changes and the rate of cell division. In guard cells, stimulus-induced elevations in $[Ca^{2+}]_{cyt}$ also show marked spatial heterogeneities (Fig. 7.2). McAinsh et al. (1992) observed that ABA-induced elevations in $[Ca^{2+}]_{cyt}$ were unevenly distributed and appeared as 'hotspots' and Ca^{2+} quiescent regions. McAinsh et al. (1995) also recorded marked spatial heterogeneities in external Ca^{2+}-induced changes in $[Ca^{2+}]_{cyt}$. Spatial heterogeneities in guard cell $[Ca^{2+}]_{cyt}$ have been reported by Gilroy et al. (1991) and Irving et al. (1992), too. It is possible that such spatial heterogeneities in guard cell $[Ca^{2+}]_{cyt}$ elevations result from (1) differential accessibility of the stimulus to only a subset of the signalling machinery or (2) the non-uniform distribution of the intracellular signalling machinery (McAinsh and Hetherington 1998; Ng and McAinsh 2003). These observations suggest the potential for encoding specificity in the form of localized increases in $[Ca^{2+}]_{cyt}$. In animal cells, global Ca^{2+} signals such as Ca^{2+} waves result from a series of localized 'elemental events' termed 'quarks', 'blips', 'puffs' and 'sparks' (Berridge et al. 2000; Bootman et al. 2001). It is tempting to suggest that the localized Ca^{2+} hotspots observed in guard cells represent elemental events similar to those of animal cells and also *Fucus*. Due to the spatial and temporal resolution used in studies of guard cell $[Ca^{2+}]_{cyt}$, however, it is likely that these localized elevations

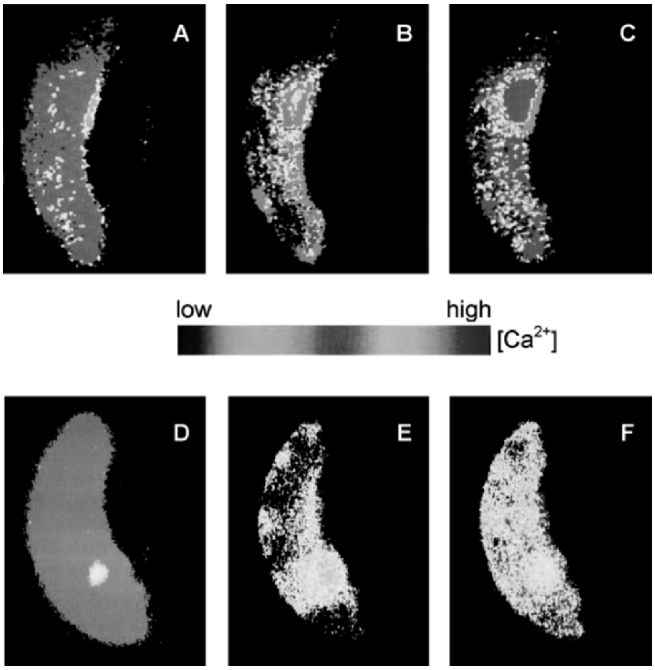


Fig. 7.2 Spatial heterogeneities in external Ca^{2+}- and ABA-induced changes in guard cell $[Ca^{2+}]_{cyt}$. The distribution of 'resting' $[Ca^{2+}]_{cyt}$ (**a**, **d**) and stimulus-induced changes in $[Ca^{2+}]_{cyt}$ were monitored in guard cells of *C. communis* approximately 15 s (**b**, **e**) and 2 min (**c**, **f**) following the addition of 100 nM ABA (**b**, **c**) and 1 mM external Ca^{2+} (**e**, **f**). $[Ca^{2+}]_{cyt}$ levels are indicated by different levels of *shading* (*dark grey* low $[Ca^{2+}]_{cyt}$, *light grey* high $[Ca^{2+}]_{cyt}$). These data suggest the capacity to encode specificity in the guard cell Ca^{2+} signal in the form of localized increases in $[Ca^{2+}]_{cyt}$ (reproduced, with permission, from McAinsh et al. 1992, 1995)

in $[Ca^{2+}]_{cyt}$ represent longer transients in $[Ca^{2+}]_{cyt}$, in contrast to the unitary Ca^{2+} elevations documented in *Fucus* in response to osmotic stress.

Temporal heterogeneities in $[Ca^{2+}]_{cyt}$, including transients, spikes and oscillations, have also been shown to be important in encoding specificity in the Ca^{2+} signal in animals (Berridge et al. 2003). In mammalian cells, the pattern of stimulus-induced oscillations in $[Ca^{2+}]_{cyt}$ is dependent on cell type as well as on the strength and nature of the stimulus. For example, it has been shown that the amplitude and duration of Ca^{2+} signals differentially control the activation of transcriptional regulators (Dolmetsch et al. 1998). In addition, work in pancreatic acinar cells has demonstrated that agonist-induced Ca^{2+} spikes in the micromolar range are necessary for the induction of exocytosis whereas Ca^{2+} spikes in the submicromolar range are associated with the activation of luminal and basal ion channels (Ito et al. 1997). Therefore, it has been proposed that signalling information is encoded both in the amplitude (also termed 'analogue'-encoded information) and in the frequency (also termed 'digital'-encoded information) of stimulus-induced

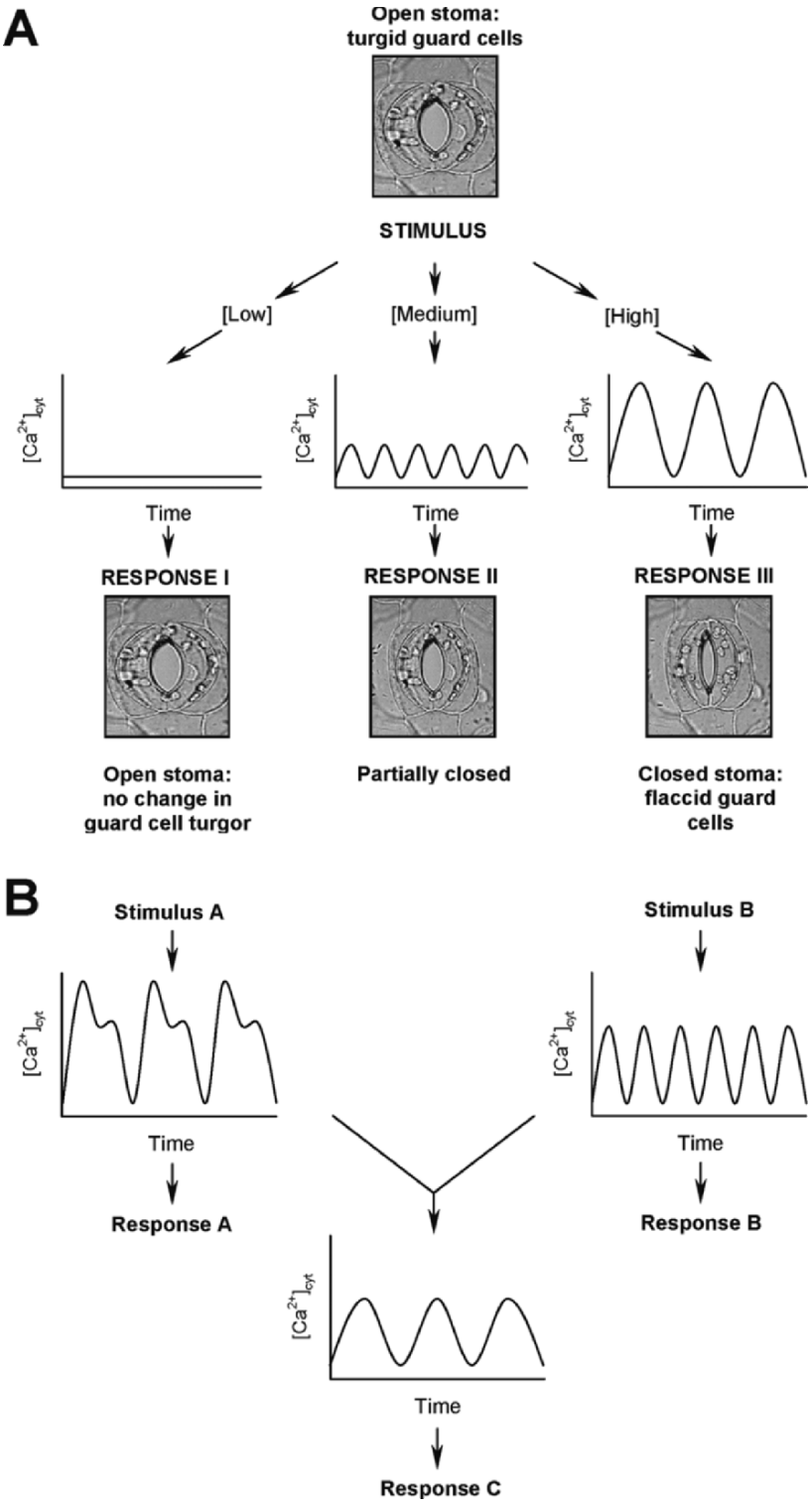
oscillations in $[Ca^{2+}]_{cyt}$ (Berridge et al. 1988, 2003; Fewtrell 1993; McAinsh et al. 1997; McAinsh and Hetherington 1998; Evans et al. 2001; Ng et al. 2001). For example, stimulus X may induce a pattern of oscillations with amplitude=A and frequency=F giving response X′ whereas a different stimulus, Y, may induce a completely different pattern of oscillations with amplitude=0.5A and frequency=2F to give a different response Y′. The mechanisms of generation and maintenance of these complex kinetics include both positive and negative feedback, often invoking the release of Ca^{2+} from intracellular stores through the action of additional second messengers such as inositol (1,4,5)-trisphosphate, and fluxes of Ca^{2+} across the plasma membrane or between intracellular stores (Berridge et al. 2000, 2003; Bootman et al. 2001).

As discussed above, temporal heterogeneities in stimulus-induced $[Ca^{2+}]_{cyt}$ changes in the form of transients and oscillations have been observed in a number of different plant cells (Holdaway-Clark and Hepler 2003; Shaw and Long 2003), including guard cells (Fan et al. 2004). Figure 7.3 shows a schematic representation of the mechanism by which guard cells may differentiate between different Ca^{2+}-mobilizing stimuli and/or different strengths of the same stimulus through the generation of specific Ca^{2+} signatures (McAinsh and Hetherington 1998; Evans et al. 2001). Here, differences in the pattern of guard cell $[Ca^{2+}]_{cyt}$ oscillations (i.e. the period, frequency and amplitude) encode information about both the nature and strength of the stimulus and will, in turn, dictate the resultant steady-state stomatal aperture (Fig. 7.3a). In addition, this provides a mechanism whereby guard cells are able to integrate signalling information from a number of stimuli which induce oscillations in $[Ca^{2+}]_{cyt}$ acting in concert when formulating the final stomatal aperture for a given set of environmental conditions through the generation of a novel calcium signature (Fig. 7.3b).

7.4.1 Guard Cell Ca^{2+} Signatures: Correlative Evidence

Hetherington and Woodward (2003) have proposed it most appropriate to describe the signalling machinery by which plant cells respond to stimuli as a network, and have positioned a change in $[Ca^{2+}]_{cyt}$ as a key hub in the guard cell signalling network, highlighting further the necessity for specificity in guard cell Ca^{2+} signalling. Oscillations in guard cell $[Ca^{2+}]_{cyt}$ have been reported in response to various stimuli including ABA, external Ca^{2+}, H_2O_2, cold, CO_2, hyperpolarization, cyclic ADP-ribose, sphingosine-1-phosphate and pathogenic elicitors (Table 7.1).

Fig. 7.3 Encoding signalling information in guard cell Ca^{2+} signatures. **a** The strength of the stimulus has been correlated directly with the pattern of $[Ca^{2+}]_{cyt}$ oscillations (i.e. the period, frequency and amplitude) which, in turn, dictates the resultant steady-state stomatal aperture. **b** Guard cells are able to integrate signalling information from a number of stimuli which induce oscillations simultaneously, to generate a novel Ca^{2+} signature when formulating the final stomatal aperture. Reproduced, with permission, from Evans et al. (2001)


A
Open stoma:
turgid guard cells
STIMULUS
[Low]
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[High]
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Time
RESPONSE I
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Open stoma:
no change in
guard cell turgor
Partially closed
Closed stoma:
flaccid guard
cells
B
Stimulus A
Stimulus B
Response A
Response B
Response C

Table 7.1 Stimulus-induced oscillations in guard cell $[Ca^{2+}]_{cyt}$

Stimulus	Species	Characteristics	Selected references
ABA	*C. communis*	10 nM: period, 11.0±0.9 min; amplitude, 200–600 nM 1 μM: period, 18.0±0.9 min; amplitude, 200–600 nM	Staxen et al. (1999)
	A. thaliana	Transients: period, 468±41 s; amplitude, ~500 nM Oscillations: period, 333±35 s; amplitude, ~500 nM	Allen et al. (1999a, 2000, 2001)
External Ca^{2+}	*C. communis*	0.1 mM: period, 8.3±0.8 min; amplitude, 300–560 nM 1.0 mM: period, 13.6±0.6 min; amplitude, 400–850 nM	McAinsh et al. (1995), Hetherington et al. (1998), Staxen et al. (1999)
	A. thaliana	750 μM: period, 5.8±0.1 min; amplitude, <60 nM 10 mM: period, 6.7±0.2 min; amplitude, >120 nM	Allen et al. (1999b)
	A. thaliana	1 mM: period, 161±20 s; amplitude, ~160 nM 10 mM: period, 396±23 s; amplitude, ~1,020 nM	Allen et al. (1999b, 2000, 2001), Hugouvieux et al. (2001), Jung et al. (2002), Klusener et al. (2002), Kwak et al. (2002, 2003)
Caged Ca^{2+}	*C. communis*	Period, 4.5±0.3 min	McAinsh et al. (1995)
H_2O_2	*A. thaliana*	Repetitive transients: amplitude, ~700 nM	Allen et al. (2000)
Cold	*A. thaliana*	Repetitive transients: period, 154±11 s; amplitude, ~125 nM	Allen et al. (2000)
Hyperpolarization	*V. faba*	Membrane hyperpolarization: oscillations and 'waves'	Grabov and Blatt (1998)
	C. communis	Low $[K^+]_{ext}$: oscillations in 30% cells	Staxen et al. (1999)
	A. thaliana	Low $[K^+]_{ext}$ transients and oscillations	Allen et al. (1999a, 2000, 2001)
Cyclic ADP-ribose	*C. communis*	Period, ~3.75 min; amplitude, ~200 nM	Leckie et al. (1998)
Sphingosine-1-phosphate	*C. communis*	6 μM: period, 3.8±0.4 min; amplitude, 30–50 nM 50 μM: period, 2.8±0.2 min; amplitude, 50–100 nM	Ng et al. (2001)

(*Continued*)

Table 7.1 Stimulus-induced oscillations in guard cell $[Ca^{2+}]_{cyt}$—Continued

Stimulus	Species	Characteristics	Selected references
CO_2	*A. thaliana*	115 ppm: period, 1.55±0.16 per 10 min; relative amplitude, 0.26±0.02 508 ppm: period, 0.95±0.13 per 10 min; relative amplitude, 0.21±0.02	Young et al. (2006)
Pathogenic elicitors	*A. thaliana*	Yeast elicitor: transient period, 5.74±1.52 min; relative amplitude, 0.45±0.10 Chitosan: transient period, 5.26±0.32 min; relative amplitude, 0.66±0.28	Klusener et al. (2002)

McAinsh and co-workers (McAinsh et al. 1995; Hetherington et al. 1998; Staxen et al. 1999) have performed a series of studies on guard cells of *Commelina communis* 'loaded' with the fluorescent Ca^{2+}-sensitive indicator fura-2, aimed at determining whether such oscillations in $[Ca^{2+}]_{cyt}$ encode the necessary signalling information required for guard cells to distinguish between different stimuli and between different strengths of a given stimulus, as in animals, or whether they are simply phenomenological. The authors showed that there is a direct relationship between the pattern of external Ca^{2+}-induced oscillations in guard cell $[Ca^{2+}]_{cyt}$, the strength of the external Ca^{2+} stimulus and the resultant steady-state stomatal aperture (Fig. 7.4a, b); 0.1 mM external Ca^{2+} induced symmetrical oscillations (amplitude, 300–560 nM; mean period, 8.3 min) whereas 1.0 mM external Ca^{2+} induced oscillations which were asymmetrical in character (amplitude, 400–850 nM; mean period, 13.6 min), resulting in an approximate 12.7 and 41.6% reduction in steady-state stomatal aperture respectively (McAinsh et al. 1995). They also observed a direct relationship between the pattern of ABA-induced oscillations in guard cell $[Ca^{2+}]_{cyt}$, the concentration of ABA and the magnitude of ABA-induced stomatal closure (Staxen et al. 1999; Fig. 7.4c, d). In both cases, the continued presence of the stimulus was required to maintain the oscillations in $[Ca^{2+}]_{cyt}$ (McAinsh et al. 1995; Staxen et al. 1999), and it was possible to reversibly switch between the different oscillatory patterns by changing the strength of the stimulus within the range inducing oscillations (Fig. 7.4e).

Allen and co-workers (Allen et al. 1999a, 2000, 2001) have addressed this question in *Arabidopsis*, using the Ca^{2+}-sensitive fluorescent-protein-based cameleon reporter to monitor stimulus-induced changes in $[Ca^{2+}]_{cyt}$. They were able to establish a relationship between the strength of the stimulus and the pattern of changes in guard cell $[Ca^{2+}]_{cyt}$ similar to that observed in *C. communis* using this approach; 1 mM external Ca^{2+} induced oscillations

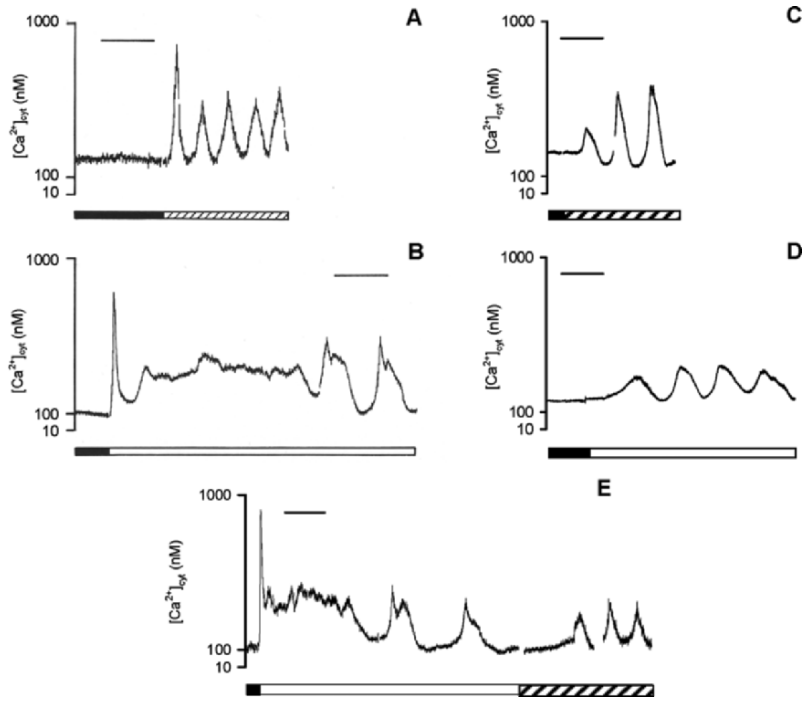

Fig. 7.4 External Ca^{2+}- and ABA-induced oscillations in guard cell $[Ca^{2+}]_{cyt}$. 'Resting' $[Ca^{2+}]_{cyt}$ (*solid bars*) and stimulus-induced changes in $[Ca^{2+}]_{cyt}$ were monitored in guard cells of *C. communis* in response to external Ca^{2+} (**a**, **b**, **e**: *hatched bars* 0.1 mM, *open bars* 1.0 mM) and ABA (**c**, **d**: *hatched bars* 10 nM, *open bars* 1.0 μM). Bars=10 min. These data suggest the capacity to encode specificity in the guard cell Ca^{2+} signal in the form of temporal heterogeneities in changes in $[Ca^{2+}]_{cyt}$. Reproduced, with permission, from McAinsh et al. (1995) and Staxen et al. (1999)

in $[Ca^{2+}]_{cyt}$ with a mean amplitude of 160 nM and a mean period of 2.6 min whereas 10 mM external Ca^{2+} induced oscillations with a mean amplitude of 1,020 nM and a mean period of 6.6 min (Allen et al. 2000) to 8.2 min (Allen et al. 1999a). These data correlate well with those obtained by Allen and co-workers using fura-2 to monitor guard cell $[Ca^{2+}]_{cyt}$ dynamics in *Arabidopsis* (Allen et al. 1999b). Comparing *Arabidopsis* with *C. communis*, the differences in the range of external Ca^{2+} concentrations inducing oscillations in guard cell $[Ca^{2+}]_{cyt}$, and in the temporal dynamics of the oscillations are consistent with established species-dependent variations in stomatal responses to external Ca^{2+} and ABA (Prokic et al. 2006). Allen and co-workers have also observed ABA-induced $[Ca^{2+}]_{cyt}$ transients in the guard cells of a range of *Arabidopsis* ecotypes with either a mean amplitude of 500 nM and a mean period of 7.8 min or with no obvious periodicity (Allen et al. 2001, 2002; Hugouvieux et al. 2001; Jung et al. 2002; Klusener et al. 2002; Kwak et al. 2002, 2003). Taken together, the data from *C. communis* and *Arabidopsis* suggest that oscillations in guard cell $[Ca^{2+}]_{cyt}$ have the potential to encode the signalling

information required for guard cells to produce a graded response to different stimuli perceived individually.

McAinsh and co-workers have also demonstrated the potential of oscillations in $[Ca^{2+}]_{cyt}$ to integrate signalling information from a number of stimuli perceived simultaneously by guard cells through the generation of a novel Ca^{2+} signature characteristic of the composite stimulus (Hetherington et al. 1998). Challenge of guard cells exhibiting external Ca^{2+}-induced oscillations in $[Ca^{2+}]_{cyt}$ with another stimulus (e.g. external $[K^+]$, ABA or osmotic stress) resulted in the modulation of the established guard cell Ca^{2+} signature (Fig. 7.5). Importantly, in terms of the encryption of signalling information, the change in the pattern of the oscillations was dependent both on the type (Fig. 7.5a, b) and strength (Fig. 7.5c) of the interacting stimuli. Therefore, oscillations in guard cell $[Ca^{2+}]_{cyt}$ appear to represent a dynamic signalling mechanism capable of encoding information in the kinetics of the guard cell Ca^{2+} signature concerning the strength and type of stimuli experienced by these cells, whether acting individually or in concert, when formulating the optimal stomatal aperture under a specific set of environmental conditions.

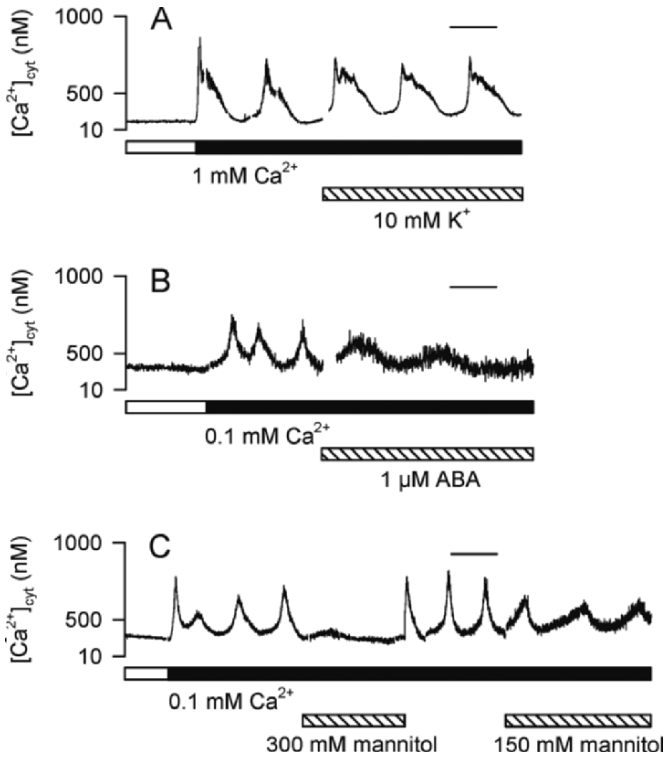

Fig. 7.5 Multiple stimuli generate novel Ca^{2+} signatures. Modulation of 1 mM or 0.1 mM external Ca^{2+}-induced oscillations in $[Ca^{2+}]_{cyt}$ in guard cells of *C. communis* by **a** $[K^+]_{ext}$, **b** ABA and **c** osmotic stress. Bars=10 min. Reproduced, with permission, from Hetherington et al. (1998)

7.4.2 Guard Cell Ca^{2+} Signatures: Evidence for a Causal Relationship

Although the findings of McAinsh and co-workers (McAinsh et al. 1995; Hetherington et al. 1998; Staxen et al. 1999) and Allen and co-workers (Allen et al. 1999a, b, 2000, 2001) illustrate clearly the potential for oscillations in $[Ca^{2+}]_{cyt}$ to encode signalling information, supporting a role for $[Ca^{2+}]_{cyt}$ oscillations in the control of stomatal aperture, they do not establish any causal relationship. To test this hypothesis, Allen and co-workers adopted a mutant approach in *Arabidopsis* to establish a stronger link between oscillations in guard cell $[Ca^{2+}]_{cyt}$ and the control of stomatal aperture (Allen et al. 2000). As discussed previously (White 2000; Sanders et al. 2002; Hetherington and Brownlee 2004; see also Chap. 6 by Blatt et al., this book), both Ca^{2+} influx and Ca^{2+} release from endomembrane stores contribute to the generation of stimulus-induced increases in $[Ca^{2+}]_{cyt}$ in plants. Consequently, impairment of endomembrane Ca^{2+} transport could provide a direct approach for dissecting guard cell Ca^{2+} signals. The de-etiolated 3 (*det3*) mutant of *Arabidopsis* exhibits a 60% reduction in expression of the C-subunit of the V-type H^+ ATPase (Schumacher et al. 1999). Since in many organisms Ca^{2+} sequestration into endomembrane stores is H^+ gradient dependent (Rooney et al. 1994; Xie et al. 1996; Hirschi 1999; Sze et al. 1999; Camello-Almaraz et al. 2000), the authors therefore hypothesized that endomembrane de-energization in *det3* could affect guard cell Ca^{2+} signalling. To this end, Allen et al. (2000) investigated the effect of *det3* on the oscillations in guard cell $[Ca^{2+}]_{cyt}$ and stomatal closure observed in response to external Ca^{2+}, ABA, H_2O_2 and cold. In wild-type plants, they observed oscillations in guard cell $[Ca^{2+}]_{cyt}$ of differing amplitudes/frequencies and long-term steady-state stomatal closure in response to all four stimuli. In *det3*, by contrast, only ABA and cold elicited oscillations in guard cell $[Ca^{2+}]_{cyt}$ with similar kinetics to those of wild-type guard cells, and induced the same long-term steady-state stomatal aperture as in wild-type plants. *det3* guard cells exhibited prolonged increases in $[Ca^{2+}]_{cyt}$ which failed to oscillate in response to external Ca^{2+} and H_2O_2, and stomatal closure was abolished. Importantly, the total external Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ integrated over a 30-min period was higher in the *det3* than in wild-type guard cells, demonstrating clearly that the reason why stomata of *det3* failed to close in response to external Ca^{2+} was an inability to generate oscillations in guard cell $[Ca^{2+}]_{cyt}$, rather than an inability to increase $[Ca^{2+}]_{cyt}$ per se. In addition, the data suggest that, in guard cells, disruption of oscillations has a negative effect on physiological responses, as is the case in animal cells (De Koninck and Schulman 1998; Dolmetsch et al. 1998; Li et al. 1998).

To confirm this, Allen et al. (2000) used a 'Ca^{2+} clamp' protocol to modify guard cell $[Ca^{2+}]_{cyt}$, and observed the resultant effect on long-term steady-state stomatal closure. The influx of external Ca^{2+} into guard cells is enhanced by plasma membrane hyperpolarization (Gilroy et al. 1991; Grabov and Blatt 1998; Allen et al. 2000; Hamilton et al. 2000). Therefore, Allen et al. (2000) used repeated changes between high (100 mM) KCl buffer (depolarizing

buffer) and a low (0.1 mM) KCl buffer containing 10 mM Ca^{2+} (hyperpolarizing buffer) to impose prolonged increases or oscillations in guard cell $[Ca^{2+}]_{cyt}$. They showed that imposing prolonged increases in $[Ca^{2+}]_{cyt}$ in wild-type guard cells, similar to the external Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increases observed in *det3*, failed to elicit long-term steady-state stomatal closure. By contrast, imposing oscillations in $[Ca^{2+}]_{cyt}$ in *det3* guard cells rescued external Ca^{2+}-induced stomatal closure, supporting the hypothesis that $[Ca^{2+}]_{cyt}$ oscillations are required for physiological responses in guard cells. The excellent correlation between the stimuli for which $[Ca^{2+}]_{cyt}$ oscillations were disrupted in *det3* and for which stomatal closure was also abolished in the mutant provides strong evidence that $[Ca^{2+}]_{cyt}$ oscillations are required in the signalling pathway leading to stomatal closure.

7.4.3 Guard Cell Ca^{2+} Signatures: the Role of Oscillations

Allen and co-workers (Allen et al. 2001) also used the Ca^{2+} clamp protocol (Allen et al. 2000) to investigate those parameters of the guard cell $[Ca^{2+}]_{cyt}$ oscillation kinetics which control stomatal responses. They monitored how different patterns of oscillation impacted on the steady-state apertures measured 3 h after the start of the $[Ca^{2+}]_{cyt}$ oscillations (Allen et al. 2001). They observed that three transients generated by exchanges between depolarizing and hyperpolarizing buffers every 5 min, creating oscillations in guard cell $[Ca^{2+}]_{cyt}$ with a fixed hyperpolarization to hyperpolarization period of 10 min, were sufficient to elicit near-maximal stomatal closure (54±2.2%). Adopting this as a standard treatment, they showed that an oscillation of 10-min period and 5-min duration elicited the greatest decrease in steady-state stomatal aperture and that reducing the amplitude of the $[Ca^{2+}]_{cyt}$ oscillations also reduced steady-state stomatal closure. Importantly, changes in guard cell $[Ca^{2+}]_{cyt}$ outside of this window of oscillation parameters resulted in immediate short-term stomatal closure but failed to evoke steady-state changes in aperture. On the basis of these data, Allen and co-workers suggested that $[Ca^{2+}]_{cyt}$ controls stomatal closure through two mechanisms: short-term 'Ca^{2+}-reactive' closure, which occurs rapidly when $[Ca^{2+}]_{cyt}$ is elevated, and long-term steady-state closure, which is 'Ca^{2+} programmed' by $[Ca^{2+}]_{cyt}$ oscillations within a defined range of frequency, transient number, duration and amplitude.

In order to explore the significance of their observations further, Allen and co-workers again adopted a mutant approach to investigate the physiological basis for the differential response of stomata of wild-type *Arabidopsis* and of the recessive ABA-insensitive *Arabidopsis* mutant *gca2* to external Ca^{2+} and ABA (Allen et al. 2001). They observed that, in wild-type plants, the kinetics of external Ca^{2+}- and ABA-induced oscillations in guard cell $[Ca^{2+}]_{cyt}$ both fell within the window of parameters resulting in long-term steady-state closure, whereas the period and duration of $[Ca^{2+}]_{cyt}$ oscillations were significantly

shorter in *gca2* such that steady-state stomatal closure was abolished. Interestingly, when the Ca^{2+} clamp protocol (Allen et al. 2000) was used to impose oscillations in guard cell $[Ca^{2+}]_{cyt}$ in wild-type plants with the mean kinetics of those observed in *gca2*, no long-term steady-state stomatal closure was observed, whereas the imposition of oscillations in guard cell $[Ca^{2+}]_{cyt}$ with a period of 10 min rescued long-term steady-state stomatal closure in *gca2*. Overall, these data show that *gca2* guard cells remain competent to respond to $[Ca^{2+}]_{cyt}$ oscillations if the transient duration and frequency are within the range required to elicit long-term steady-state stomatal closure, confirming the important role $[Ca^{2+}]_{cyt}$ oscillations in guard cells play in the control of stomatal aperture, together with their ability to encode information pertaining to long-term steady-state stomatal closure.

However, Hetherington and Brownlee (2004) have urged caution when making generalizations about the role of $[Ca^{2+}]_{cyt}$ oscillations in guard cells. Specifically, they highlight reports that ABA, elevated $[CO_2]$, and H_2O_2 can all induce stomatal closure in the absence of oscillations in guard cell $[Ca^{2+}]_{cyt}$ (McAinsh et al. 1990, 1992, 1996; Gilroy et al. 1991; Webb et al. 1996) and of spontaneous $[Ca^{2+}]_{cyt}$ transients in guard cells which do not result in stomatal closure (Klusener et al. 2002). They acknowledge that, in many of these studies, the duration of the measurements may have been insufficient for a steady-state stomatal aperture to occur and, hence, guard cell $[Ca^{2+}]_{cyt}$ oscillations to be achieved, or that the kinetics of spontaneous oscillations may fall outside of the widow required for long-term steady-state stomatal closure. Nevertheless, they note the need for further explanation of these apparent anomalies, given that, for example, 10 mM external Ca^{2+} induces oscillations in guard cell $[Ca^{2+}]_{cyt}$ with a period similar to that of spontaneous $[Ca^{2+}]_{cyt}$ oscillations, and that 10 mM external Ca^{2+} is known to close stomata (Allen et al. 1999a, 2000).

7.5 The Ca^{2+} Sensor Priming Model of Guard Cell Ca^{2+} Signalling

The answers to some of the issues raised by Hetherington and Brownlee (2004) may be found, at least in part, in a recent study of CO_2 signalling in guard cells. Young et al. (2006) have demonstrated the presence of CO_2-induced transients in guard cell $[Ca^{2+}]_{cyt}$. They show that elevated $[CO_2]$ (508 ppm), which promotes stomatal closure, induced slow repetitive transients in $[Ca^{2+}]_{cyt}$ similar to those reported during ABA-induced stomatal closure (Grabov and Blatt 1998; Staxen et al. 1999; Allen et al. 2000, 2001) whereas low $[CO_2]$ (115 ppm), which promotes stomatal opening, induced more rapid $[Ca^{2+}]_{cyt}$ transients; shifts in $[CO_2]$ resulted in modulation of the $[Ca^{2+}]_{cyt}$ transients. This is an unexpected result in the light of previous reports of elevated $[CO_2]$-induced increases in guard cell $[Ca^{2+}]_{cyt}$ (Webb et al. 1996). In addition, treatment with BAPTA-AM to chelate internal Ca^{2+}, with no added external

Ca^{2+}, abolished both the high and low $[CO_2]$-induced $[Ca^{2+}]_{cyt}$ transients preventing long-term CO_2-induced stomatal closure and reducing low $[CO_2]$-induced stomatal opening. Furthermore, guard cells of the recessive ABA-insensitive mutant *gca2* which, as discussed above, has been shown to exhibit an aberrant pattern of ABA-induced guard cell $[Ca^{2+}]_{cyt}$ transients compared to wild-type (Allen et al. 2001), showed little change in the rate of $[CO_2]$-induced transients in guard cell $[Ca^{2+}]_{cyt}$ in response to an increase in $[CO_2]$ from 115 to 508 ppm CO_2. Elevated $[CO_2]$-induced stomatal closure was also strongly attenuated in *gca2* plants. These data establish a causal link between CO_2-induced guard cell $[Ca^{2+}]_{cyt}$ transients and the maintenance of an adjusted stomatal aperture after a $[CO_2]$ change, most notably an increase in $[CO_2]$, and suggest a differential role for $[Ca^{2+}]_{cyt}$ in CO_2-induced stomatal opening and closure responses.

Previous studies have shown that experimentally imposed $[Ca^{2+}]_{cyt}$ oscillations in guard cells trigger immediate stomatal closure (cf. the Ca^{2+}-reactive closure discussed by Allen et al. 2001), regardless of the pattern of $[Ca^{2+}]_{cyt}$ changes (Allen et al. 2001; Yang et al. 2003; Li et al. 2004). However, Young et al. (2006) did not detect any measurable short-term Ca^{2+}-reactive stomatal closure associated with the rapid guard cell $[Ca^{2+}]_{cyt}$ transients induced by low $[CO_2]$ and, indeed, it would be inappropriate for an opening stimulus such as low $[CO_2]$ to generate $[Ca^{2+}]_{cyt}$ transients in guard cells which result in stomatal closure. Therefore, the authors have proposed a new Ca^{2+} sensor priming model to explain how stimuli bring about an appropriate physiological response requiring the generation of both the correct Ca^{2+} signature (oscillations and transients in $[Ca^{2+}]_{cyt}$) and the correct signal transduction state or 'physiological address' (McAinsh et al. 1997; McAinsh and Hetherington 1998), through the modulation of Ca^{2+} sensors (Ca^{2+} sensor priming), to enable the cell to respond properly to the stimulus-induced changes in $[Ca^{2+}]_{cyt}$. On this basis, low $[CO_2]$ produces a guard cell physiological address which does not permit short-term Ca^{2+}-reactive stomatal closure to occur in response to low $[CO_2]$-induced guard cell $[Ca^{2+}]_{cyt}$ transients. Further evidence for this model comes from studies showing that guard cell anion channels can be primed for activation by changes in $[Ca^{2+}]_{cyt}$ by exposure to different levels of external Ca^{2+} (Allen et al. 2002). It will be intriguing to explore further the complex interplay between stimulus-induced Ca^{2+} signatures and the proposed stimulus-induced modulation of cellular physiological addresses, and how these contribute to stimulus specificity in the guard cell signalling network.

7.6 Decoding Ca^{2+} Signatures in Plants

It is clear that plants, and stomatal guard cells in particular, have the ability to generate stimulus-specific Ca^{2+} signatures with the potential to encode information about both the nature and strength of the stimulus. However, it is essential that they also possess the necessary machinery to decode this

information. In animal cells, several models have been proposed to explain how signalling information encrypted in transients, spikes and oscillations in $[Ca^{2+}]_{cyt}$ might be decoded, based largely on Ca^{2+}-dependent protein kinases and phosphatases (De Koninck and Schulman 1998; Goldbeter et al. 1998) as well as protein kinase C (Oancea and Meyer 1998). In plants, several families of Ca^{2+} sensors are good candidates as primary decoders of oscillations in plant $[Ca^{2+}]_{cyt}$. These include Ca^{2+}-regulated protein kinases, of which there are four families in plants (Harper et al. 2004), camodulin and its isoforms (Snedden and Fromm 2001; Yang and Poovaiah 2003) together with the calmodulin-binding proteins (Snedden and Fromm 2005), and the calcineurin-B-like proteins (CBLs) together with their interacting protein kinases (CBL-interacting protein kinase, CIPKs; Kolukisaoglu et al. 2004). Although the regulation, function and interconnections of most of these sensor proteins remain to be determined, they are likely to recognize specific Ca^{2+} signatures and relay these signals into downstream responses (Luan et al. 2002). The cytoskeleton is also emerging as a dynamic structure playing an important role in the perception and response of plant cells to stimuli (Staiger 2000; Drobak et al. 2004). In addition, Ca^{2+}-regulated proteolysis (Ransom-Hodgkins et al. 2000) and interference with protein expression at the posttranscriptional level, through targeted proteolysis and the regulation of the translation of specific mRNAs by RNA-binding proteins, may also contribute to decoding the signalling information encrypted in stimulus-induced changes in plant $[Ca^{2+}]_{cyt}$ and, specifically, guard cell Ca^{2+} signatures.

7.7 Challenging Prospects

The last 20 years have witnessed significant developments in our understanding of Ca^{2+}-based signalling in plants, including the role of oscillations in $[Ca^{2+}]_{cyt}$ and their potential to encode signalling information in oscillations in $[Ca^{2+}]_{cyt}$ within a defined range of frequency, transient number, duration and amplitude through the generation of unique Ca^{2+} signatures (McAinsh et al. 1992, 1995, 1997; McAinsh and Hetherington 1998). However, problems associated with introducing Ca^{2+} indicators into single plant cells have meant that studies of $[Ca^{2+}]_{cyt}$ oscillations have been restricted to only a few cell types, most notably root hairs, pollen tubes and stomata guard cells, and have lagged significantly behind those in animal cells (Rudd and Franklin-Tong 2001; Hetherington and Brownlee 2004). Although many different fluorescent Ca^{2+}-sensitive indicators are currently available, these are all anionic at physiological pH (Grynkiewicz et al. 1985) and, unless the extracellular pH is lowered, do not diffuse readily into cells (Wymer et al. 1997). This is not a problem in animal cells, which can be loaded using the uncharged acetoxymethyl (AM) -ester forms of indicators which diffuse across the plasma membrane into the cytosol and are hydrolysed to the anionic form by

intracellular esterases (Tsien 1981). In plants, by contrast, esterases in the cell wall (Micheli 2001) hydrolyse the AM-esters before they can enter cells and, despite attempts to inhibit extracellular esterase activity using eserine (Tretyn et al. 1997; Kuchitsu et al. 2002), the AM-ester loading technique remains of limited use in plant cells due to problems associated with the compartmentation and loss of the indicator from the cytoplasm (Bush and Jones 1987; Brownlee and Pulsford 1988). Consequently, microinjection, using either iontophoresis (McAinsh et al. 1990) or pressure (Leckie et al. 1998), has frequently been applied to load plant cells with Ca^{2+}-sensitive indicators. Although this is a robust method of loading, it is nevertheless prohibitively inefficient in small thick-walled cells such as stomatal guard cells, limiting studies of Ca^{2+} signalling in this cell type using this loading method (for example, Gilroy et al. 1990, 1991; McAinsh et al. 1990, 1992, 1995, 1996; Allan et al. 1994; Webb et al. 1996; Grabov and Blatt 1998, 1999; Leckie et al. 1998; Staxen et al. 1999; Ng et al. 2001; Webb et al. 2001; Levchenko et al. 2005).

An important development in monitoring $[Ca^{2+}]_{cyt}$ has been the use of the Ca^{2+}-sensitive fluorescent-protein-based cameleon reporters (Miyawaki et al. 1997), which provide an exciting alternative to fluorescent Ca^{2+}-sensitive indicators for monitoring $[Ca^{2+}]_{cyt}$ in plant cells. The great advantage of these is that, once successfully introduced into a plant line, they are stably expressed and can be targeted to the cytosol or specific organelles. Studies using plant cameleons have contributed significantly to our understanding of the functional significance of oscillations in $[Ca^{2+}]_{cyt}$ in plant cells and, in particular, stomatal guard cells (see above). However, the use of cameleon reporters has so far been restricted to a limited number of transformable cell types and species (e.g. Allen et al. 1999a; Iwano et al. 2004).

Recently, Bothwell et al. (2006) have developed a biolistic method using microscopic gold particles for loading Ca^{2+}-sensitive indicators into plant and algal cells, in an approach similar to that employed to transform undifferentiated plant material (Lorence and Verpoorte 2004). The method has also been used successfully to deliver Ca^{2+}-sensitive indicators into animal nervous tissue (Kettunen et al. 2002). Biolistic delivery has the advantage of being faster and technically less demanding than microinjection, may be used on a wider range of cell types than microinjection, including those which are too small or delicate to be efficiently microinjected, and may be used in species which have hitherto not been amenable to transformation. This increased ease of loading of impermeable Ca^{2+}-sensitive indicators in a variety of species will enable extrapolation of studies of oscillations in guard cell $[Ca^{2+}]_{cyt}$ performed in model species such as *Commelina communis* and *Arabidopsis* to additional species. A further advantage of this method is the ability to load cells in the intact plant, raising the possibility of in situ measurements of single cell $[Ca^{2+}]_{cyt}$ changes being routinely made in the intact plant (Roelfsema et al. 2004; Levchenko et al. 2005). The biolistic loading technique of Bothwell et al. (2006), therefore, has the potential to greatly extend the scope of studies of the physiological role of oscillations in guard

cell $[Ca^{2+}]_{cyt}$ and how these relate to other components of the guard cell signalling network (Hetherington and Woodward 2003).

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8 Circadian Rhythms in Stomata: Physiological and Molecular Aspects

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Abstract

Stomata are the major route of gas exchange between the atmosphere and the leaf interior. The size of the stomatal pore is controlled by movements of the stomatal guard cells. The guard cells close the stomatal pore to conserve water during stress. Under more favourable conditions, the stomatal movements optimise CO_2 uptake whilst minimising water loss. The movements of stomata are controlled by an extensive network of signalling pathways responding to diverse stimuli. One of the regulators of stomata is the circadian clock. We discuss the physiological mechanisms by which the clock may regulate stomatal movements, and the benefits that circadian regulation of stomatal behaviour may confer to the plant.

8.1 Introduction

Stomata are small pores on the leaf surface that are the major point of gas exchange between the leaf and the atmosphere in most plants. CO_2 enters through the pores before being fixed in photosynthesis, while water from the transpiration stream exits the plant. Each pore is delineated by a pair of guard cells, their movements regulating the stomatal aperture. Guard cells respond to a wide range of environmental signals, e.g. blue light, temperature, humidity and intercellular CO_2 concentration (C_i), by changes in turgor pressure resulting in stomatal movements (reviewed in Assmann and Wang 2001; Schroeder et al. 2001). Guard cells also respond to the physiological status of the plant, through the action of abscisic acid (ABA), indole-3-acetic acid (IAA) and cytokinins (CK; Tanaka et al. 2006). Thus, guard cells prevent water loss during periods of stress and also respond appropriately to a diverse range of environmental signals to maintain an optimal pore diameter

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enabling the plant to minimise water loss and maximise CO_2 uptake during periods of favourable conditions.

In C3 and C4 plants, stomata open during the day to enable CO_2 uptake, and are closed at night. In plants with Crassulacean Acid Metabolism (CAM), the phasing of stomatal movements is the reverse of that in C3 and C4 plants, the stomata being open for part or all of the night to enable CO_2 fixation by phospho*enol*pyruvate carboxylase (PEPC). Nocturnal stomatal opening in CAM plants conserves water due to reduced transpiration in the cool of the night because the gradient in water loss is reduced by the lower temperatures and increased relative humidity of the air (Webb 1998). Clearly, the correct timing of stomatal movements through the day–night cycle is critical for the control of plant physiology. For example, in a C3 plant, a delay in stomatal opening after dawn could result in reduced total daily carbon fixation due to insufficient atmospheric CO_2 being available to the mesophyll during the day; and extended opening after dusk could increase water loss without any increase in carbon fixation. Since appropriate timing of stomatal movements is critical for optimal photosynthesis and water relations, it is perhaps unsurprising that guard cell movements also are regulated by the circadian clock (Gorton et al. 1989; Somers et al. 1998; Dodd et al. 2004). In this chapter, we will consider the role of the circadian clock in regulating guard cell physiology, rhythmic sensitivity to environmental signals and the contribution that rhythmic guard cell movements make to the fitness of the plant.

For the benefit of the reader, in the following list are defined the main abbreviations used here. ABA, abscisic acid; *ABH1*, *ABA HYPERSENSITIVE 1*; *abi1*, *abscisic acid insensitive 1*; *AtMRP5*, *ARABIDOPSIS MULTIDRUG RESISTANCE-RELATED PROTEIN 1*; *AtRbohD*, *ARABIDOPSIS RESPIRATORY BURST OXIDASE HOMOLOGUE 5*; *AKT2/3*, *ARABIDOPSIS* K^+ *TRANSPORTER 2/3*; $[Ca^{2+}]_{cyt}$, concentration of cytosolic free calcium; *CAB2*, *CHLOROPHYLL A/B BINDING PROTEIN 2*; cADPR, cyclic adenosine diphosphate ribose; CAM, Crassulacean Acid Metabolism; *CCA1*, *CIRCADIAN CLOCK ASSOCIATED 1*; *CCR2*, *COLD*, *CIRCADIAN*, *RHYTHM 2*; C_i, intercellular CO_2 concentration; CK, cytokinin; CK2, CASEIN KINASE 2; DD, continuous darkness; *ELF3*, *EARLY FLOWERING 3*; *ELF4*, *EARLY FLOWERING 4*; FV, fast vacuolar channel; *GI*, *GIGANTEA*; *GORK*, *GUARD CELL EXPRESSED OUTWARD-RECTIFYING* K^+ *CHANNEL*; IAA, indole-3-acetic acid; $Ins(1,4,5)P_3$, inositol (1,4,5) trisphosphate; $InsP_6$, inositol hexakisphosphate; *LHY*, *LATE ELONGATED HYPOCOTYL*; LKP2, LIGHT, OXYGEN, VOLTAGE/KELCH PROTEIN 2; LL, continuous light; *LUC*, *LUCIFERASE*; NADPH, nicotinamide adenine dinucleotide phosphate; PEPC, phospho*enol*pyruvate carboxylase; *PHYB*, *PHYTOCHROME B*; *PRR*, *PSEUDO-RESPONSE REGULATOR*; PtdIns(3)P, phosphatidylinositol 3 phosphate; PtdIns(4)P, phosphatidylinositol 4 phosphate; ROS, reactive oxygen species; SV, slow vacuolar channel; *TOC1*, *TIMING OF CAB EXPRESSION 1*; *TPC1*, *TWO PORE CHANNEL 1*; VK, vacuolar K^+ channel; *ZTL*, *ZEITLUPE*.

8.2 Mechanisms of Stomatal Movements

Stomatal aperture is determined by the turgor pressure of the guard cells: it is estimated that guard cells of *Vicia faba* lose 40% of their volume during closure (Roelfsema and Hedrich 2005). Due to the arrangement of cellulose fibrils in the guard cell wall, increases in turgor pressure result in the swelling of cells along the longitudinal axis, while the overall length of the stomatal complex remains relatively unchanged (Sharpe et al. 1987; Shope et al. 2003). This change in cell shape results in the opening of the pore between the guard cells, thereby enabling gas exchange to proceed. The accumulation and efflux of ions bring about the changes in turgor pressure by altering the water potential of the cell and driving osmosis. Early hypotheses included a role for diurnal changes in starch production in driving stomatal movements, which have been now largely rejected in favour of models involving ion fluxes. Nevertheless, starch metabolism may indeed be important for stomatal functioning because, in guard cells of *Arabidopsis*, there is strong rhythmic control of carbohydrate metabolism, with starch synthesis occurring during the day and remobilisation at night (Stadler et al. 2003).

A complex series of ion movements at both the plasma membrane and the tonoplast contributes to changes in stomatal aperture (for detailed reviews of ion transport models, see Assmann 1993; Blatt 2000; Schroeder et al. 2001; Fan et al. 2004). Stomatal opening in response to blue light, for example, is initiated by the activation of a plasma membrane H^+-ATPase, with H^+ efflux from the cytosol resulting in hyperpolarisation of the plasma membrane to around –200 mV. Hyperpolarisation activates voltage-gated inward-rectifying K^+ channels in the plasma membrane, enabling K^+ to flow down the electrochemical gradient to the cytosol. Cl^- and NO_3^- are taken up to counterbalance the K^+ charge, a role fulfilled also by the increasing concentrations of malate^{2-} produced from CO_2 fixation by PEPC. K^+, Cl^- and malate^{2-} are accumulated in the vacuole, though the routes for entry of these ions across the tonoplast are unclear. Accumulation of these solutes in the vacuole results in an osmotic gradient for water uptake, causing the turgor increase required for stomatal opening.

Stomatal closure in response to ABA, for example, requires two distinct processes: there must be an efflux of ions and water from the vacuole to the cytosol across the tonoplast, and similar fluxes from the cytosol to the apoplast across the plasma membrane. ABA induces an increase in the concentration of cytosolic free calcium ($[Ca^{2+}]_{cyt}$) in the guard cell, and this appears to be critical for both of these events (McAinsh et al. 1990; Staxén et al. 1999; Webb et al. 2001). K^+ exits the vacuole via the vacuolar K^+ (VK) channel and possibly the fast vacuolar (FV) and the slow vacuolar (SV) channels (Ward and Schroeder 1994; Allen and Sanders 1996). The SV channel has been cloned and named *TWO PORE CHANNEL 1* (*TPC1*; Peiter et al. 2005). VK is voltage insensitive and activated by an increase in $[Ca^{2+}]_{cyt}$. Tonoplast

depolarisation due to VK activation coupled with an increase in $[Ca^{2+}]_{cyt}$ are considered to activate SV/TPC1 (Ward and Schroeder 1994; Allen and Sanders 1996). A route for anion efflux across the tonoplast has not been identified. K^+ efflux across the plasma membrane requires depolarisation of the plasma membrane to values positive of the reversal potential for K^+ (typically around −120 mV). A number of events, activated at least in part by an increase in $[Ca^{2+}]_{cyt}$, are responsible for depolarising the plasma membrane. Increases in $[Ca^{2+}]_{cyt}$ in response to ABA and other stimuli inhibit the H^+-ATPase, thereby preventing further hyperpolarisation (Kinoshita et al. 1995), and inhibit also the inward-rectifying K^+ channel, thereby preventing further K^+ influx (Grabov and Blatt 1997). Critically, ABA – through Ca^{2+}-dependent and -independent routes – also activates fast and slow anion channels that enable the efflux of Cl^- and $malate^{2-}$, this making a major contribution to plasma membrane depolarisation (Hedrich et al. 1990; Levchenko et al. 2005). The slow anion channel is voltage insensitive across a wide range of membrane potentials, enabling sustained activation of the anion channel resulting in prolonged depolarisation. At potentials positive of about −120 mV, the outward-rectifying K^+ channel is activated, facilitating K^+ efflux. Genetic evidence for the role of outward-rectifying K^+ channels in stomatal closure comes from knockout of an *Arabidopsis* outward-rectifying K^+ channel (*GUARD CELL EXPRESSED OUTWARD-RECTIFYING K⁺ CHANNEL*, *GORK*). Disruption of *GORK* resulted in complete inhibition of K^+ efflux from guard cells, and impaired stomatal closure in response to both darkness and ABA (Hosy et al. 2003).

Stomatal movements require coordination of many ion channel activities on the tonoplast and plasma membrane. This is brought about by a complex signalling network that includes changes in cytosolic pH, changes in $[Ca^{2+}]_{cyt}$ and the activation/inactivation of protein phosphatases 2C (PP2C; for reviews, see MacRobbie 1998; Hetherington 2001). ABA causes increases in $[Ca^{2+}]_{cyt}$ by activating Ca^{2+} influx across the plasma membrane through hyperpolarisation activated Ca^{2+} channels (Hamilton et al. 2000) and by release from internal stores. Release of Ca^{2+} from internal stores is mediated by Ca^{2+}-induced Ca^{2+} release, possibly through SV/TPC1 (Ward and Schroeder 1994; Peiter et al. 2005), and by second messengers such as cyclic adenosine diphosphate ribose (cADPR; Leckie et al. 1998), inositol (1,4,5) trisphosphate ($Ins(1,4,5)P_3$; Gilroy et al. 1990), inositol hexakisphosphate ($InsP_6$; Lemtiri-Chlieh et al. 2003) and, potentially, sphingosine-1-phosphate (Ng et al. 2001). Nitric oxide (NO; Garcia-Mata et al. 2003) as well as reactive oxygen species (ROS) such as H_2O_2 (Bright et al. 2006) may contribute to increases in $[Ca^{2+}]_{cyt}$ through multiple effects. Downstream responses to increased $[Ca^{2+}]_{cyt}$ are complex due to spatial and temporal heterogeneity in the pattern of $[Ca^{2+}]_{cyt}$ increases. Multiple stimuli, including external Ca^{2+}, ROS, pathogenic elicitors, CO_2 and ABA, can cause oscillatory or repeated transient increases in $[Ca^{2+}]_{cyt}$ (McAinsh et al. 1995; Staxén et al. 1999; Klusener et al. 2002; Young et al. 2006). It is thought the period of the

oscillations of $[Ca^{2+}]_{cyt}$ may encode information about stimulus strength because the period increases at higher concentrations of the external stimulus (McAinsh et al. 1995; Staxén et al. 1999; Young et al. 2006). There may be two responses to increased $[Ca^{2+}]_{cyt}$, the first due to the initial increase in $[Ca^{2+}]_{cyt}$ that causes ion efflux, leading to rapid closure. A second response is the regulation of the final steady-state aperture, which appears more complex because this is strongly dependent on the frequency and amplitude of oscillations of $[Ca^{2+}]_{cyt}$ (Allen et al. 2001).

Another effect of ABA is to raise the cytosolic pH, through a mechanism that is currently unknown (Gehring et al. 1990; Suhita et al. 2004). Nevertheless, ABA-induced pH increases activate outward-rectifying K^+ channels at the plasma membrane, promoting K^+ efflux and stomatal closure, and may also inhibit K^+ influx. Buffering cytosolic pH prevents ABA-induced activation of the outward K^+-channels (Blatt and Armstrong 1993). These data demonstrate that, although it is not yet known how pH exerts its effects on ion channels, pH is likely to be a central regulator of ABA signalling.

PP2C activity is another potent regulator of ion transport in stomatal guard cells. Genetic evidence for the role of PP2C in ABA signalling comes from the *abscisic acid insensitive 1-1* (*abi1-1*) and *abi2-1* mutations. *abi1-1* and *abi2-1* are dominant mutations causing ABA-insensitivity in a wide range of ABA responses (Leung and Giraudat 1998). The mutations have been shown to act at multiple points in the ABA-signalling pathway in guard cells, affecting ABA-induced increases in ROS and $[Ca^{2+}]_{cyt}$ as well as interfering with Ca^{2+} regulation of ion channel activity (Schroeder et al. 2001). ABI1 and ABI2 are partially redundant negative regulators of ABA signalling that are thought to regulate multiple processes (Gosti et al. 1999; Merlot et al. 2001). ABA signalling proceeds by inhibition of PP2C activity (Zhang et al. 2004). PP2C is inactivated and tethered to the plasma membrane by phosphatidic acid produced by ABA-induced activation of phospholipase D (Zhang et al. 2004).

A number of other signalling components have been identified that can regulate guard cell closure. These include other protein phosphatases, protein kinases (presumably at least some of these act to counter PP2C activity), an mRNA cap binding protein (ABA HYPERSENSITIVE 1; ABH1), a putative heterotrimeric G-protein subunit and Rho-related GTPases (Hugouvieux et al. 2001; Lemichez et al. 2001; Schroeder et al. 2001; Wang et al. 2001). However, the involvement of $[Ca^{2+}]_{cyt}$ in signalling pathways downstream of a number of closing stimuli places Ca^{2+} signalling at a central point in the guard cell signalling network, rather than as a component of completely distinct signalling pathways (Hetherington and Woodward 2003). The involvement of a common signalling component in the transduction of multiple stimuli might explain how guard cells integrate many signals to optimise the aperture of the stomatal pore. Information originating from multiple stimuli could be integrated by a change in the pattern of oscillations of $[Ca^{2+}]_{cyt}$. The shape, period and amplitude of the guard cell $[Ca^{2+}]_{cyt}$ oscillation is related to the concentration and type of stimulus (McAinsh et al. 1995), and

simultaneous multiple stimulation results in novel oscillatory patterns of $[Ca^{2+}]_{cyt}$ (Hetherington et al. 1998).

8.3 The Circadian Clock

Much has been discovered about the mechanisms by which extracellular signals regulate stomatal movements but, importantly, the circadian clock also controls stomatal aperture. The circadian clock is an internal timekeeper that enables synchronisation of molecular, physiological and metabolic events throughout the day (see Salomé and McClung 2004; Más 2005; Gardner et al. 2006 for reviews). Circadian rhythms have a period of approximately 24 h, are entrained by light–dark and temperature cycles to synchronise cellular events with rhythmic changes in the environment and, once entrained, can persist under constant conditions (Millar 2004). In contrast to most other biological processes, the oscillator is relatively insensitive to temperature, meaning that the period of the oscillator shows limited variation under a certain range of ambient temperature conditions. This is known as temperature compensation (Gould et al. 2006). Circadian clocks have evolved independently at least four times, which suggests that the clock confers a selective advantage (Young and Kay 2001). It has recently been demonstrated that, in plants, a clock period matched to that of the environment confers advantage by increasing chlorophyll content, CO_2 assimilation and growth (Dodd et al. 2005b).

The *Arabidopsis* circadian clock is composed of multiple negative feedback loops of transcriptional regulation (see Gardner et al. 2006 for review; Fig. 8.1). The first loop identified consists of the partially redundant MYB-like transcription factors *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*; Wang and Tobin 1998) and *LATE ELONGATED HYPOCOTYL* (*LHY*; Schaffer et al. 1998; Mizoguchi et al. 2002), and the pseudo-response regulator *TIMING OF CHLOROPHYLL A/B BINDING PROTEIN EXPRESSION 1* (*TOC1*; Strayer et al. 2000). *LHY* and *CCA1* are expressed rhythmically, and also in response to light, such that maximal transcript abundance occurs at subjective dawn. The LHY and CCA1 proteins bind the *TOC1* promoter at the evening element (EE; AAAATATCT) and inhibit *TOC1* expression (Alabadí et al. 2001). Towards dusk, LHY and CCA1 levels fall sufficiently to allow *TOC1* expression. TOC1 activates *LHY* and *CCA1* expression through an as yet unknown mechanism to complete the loop. Overexpression of either *LHY* or *CCA1* results in circadian arrhythmia, which demonstrates the requirement of the cyclic expression of these genes for correct clock function (Wang and Tobin 1998; Schaffer et al. 1998; Alabadí et al. 2001). Mutation of *TOC1* results in short period rhythms in a number of clock outputs (Millar et al. 1995; Somers et al. 1998; Strayer et al. 2000).

This simple model of the plant circadian clock is incomplete, with multiple lines of evidence suggesting that additional components are required in

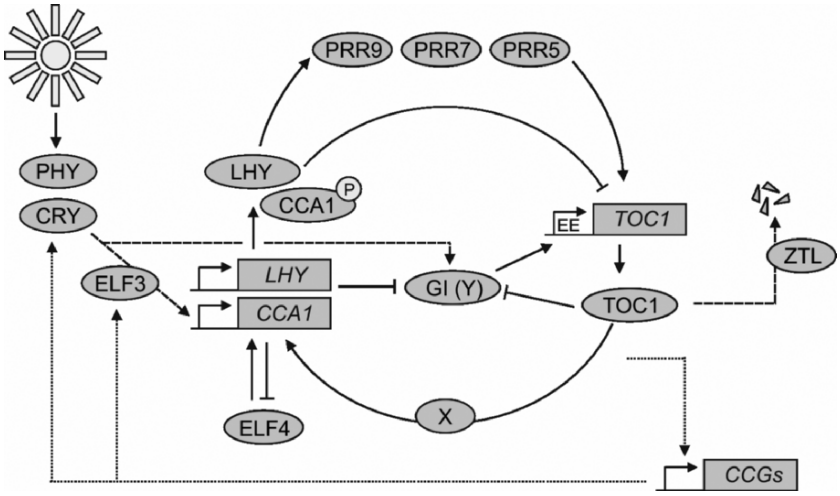

Fig. 8.1 The *Arabidopsis* circadian clock. The LHY-CCA1-TOC1-X-GI(Y) feedback loop is described in Locke et al. (2005a, b), the PRR loop in Mizuno and Nakamichi (2005) and the ELF4 loop in Kikis et al. (2005). Further explanation of the model can be found in the text. Positive interactions are shown by *arrows*, negative interactions by *perpendicular lines. Solid lines* represent genetic interactions between central oscillator components, *dotted lines* show clock output pathways and *dashed lines* show light-dependent events that modulate the central oscillator. ELF3 is involved in the gating of light signalling to the clock. ZTL is involved in the light-dependent degradation of TOC1 by the proteasome. Phosphorylation of CCA1 by CK2 is represented by P. The evening element in the TOC1 promoter is shown as EE. PHY denotes phytochromes, CRY cryptochromes, CCGs clock-regulated genes (for other abbreviations, see text)

order to explain circadian rhythms in *Arabidopsis*. For example, in a *cca1-1 lhy-21* double loss-of-function mutant, there are rhythms of the *COLD, CIRCADIAN RHYTHM::LUCIFERASE* (*CCR2::LUC*) reporter under constant light or constant dark conditions, albeit with a short period and successive dampening of the rhythm (Locke et al. 2005a). Rhythms in *EARLY FLOWERING 3* (*ELF3*) expression persist in mutants constitutively overexpressing *LHY* (Hicks et al. 1996), and the model cannot explain the observation that both mutation and overexpression of *TOC1* lead to reductions in *CCA1* and *LHY* expression (Hayama and Coupland 2003; Gardner et al. 2006).

For these reasons, the *LHY-CCA1-TOC1* model has recently been revised and expanded. Mathematical modelling of the *Arabidopsis* oscillator indicates that at least two loops are required to explain the presence of residual rhythms in a *cca1-1 lhy-21* double mutant (Locke et al. 2005a, b). The mathematical model contains an additional hypothetical component, *X*, which is introduced between *TOC1* and *LHY/CCA1*, and a second loop between *LHY/CCA1* and *TOC1* involving a hypothetical component *Y*, for which a strong candidate is *GIGANTEA* (*GI*; Locke et al. 2005a; 2006). *GI* is rapidly and transiently induced by light, its promoter contains an EE, and its expression

pattern shows a broad peak in the afternoon (Locke et al. 2005a). This closely matches the predicted profile of *Y* in the two-loop oscillator model. Overexpression or mutation of *GI* affects circadian rhythms, which suggests that *GI* is part of the central oscillator (Mizoguchi et al. 2005). Additionally, a number of other clock components have been identified that have yet to be incorporated into a mathematical description of the oscillator. These include three further members of the pseudo-response regulator family of genes (*ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 5/7/9*), in addition to *TOC1* (Makino et al. 2000; Eriksson et al. 2003; Nakamichi et al. 2005). *LUX ARRYTHMO* (*LUX*) is a MYB-like transcription factor that may form part of the clock. Unlike the *LHY* and *CCA1* MYB-like transcription factors, *LUX* forms part of the positive arm of the loop, possibly with a function similar to that of *TOC1* (Hazen et al. 2005). *ELF4* is another clock gene that forms a negative feedback loop with *CCA1* and *LHY* but functions independently of *TOC1* (Kikis et al. 2005). Post-translational regulation must also be considered for a complete model of the oscillator, because CCA1 is phosphorylated by CASEIN KINASE 2 (CK2; Sugano et al. 1998). Targeted degradation of clock proteins by the proteasome is required for oscillator function; ZEITLUPE (ZTL) and LIGHT, OXYGEN, VOLTAGE (LOV)/KELCH PROTEIN 2 (LKP2) are partially redundant F-box type proteins that mediate light-dependent degradation of clock proteins such as TOC1 (Schultz et al. 2001; Somers et al. 2004). The interactions between these elements and other central oscillator components have not been fully determined; accurate positioning of these genes within current models requires further investigation that will be aided by mathematical analysis.

8.4 Circadian Regulation of Stomatal Aperture

Circadian rhythms in guard cell movements have been recorded in a wide range of species, with period estimates ranging from 22 h (*Vicia faba*) to 26 h (*Arachis*; Stålfelt 1963; Pallas et al. 1974). Rhythms have been studied at a whole-plant level by means of infrared gas analysis (IRGA) and porometry, and at a single-cell level using epidermal strips and protoplasts (Webb 1998). Robust rhythms occur in both continuous light (LL; Gorton et al. 1989; Somers et al. 1998; Dodd et al. 2004) and continuous darkness (DD; Holmes and Klein 1986), although rhythms in continuous darkness dampen considerably after the first two cycles (Holmes and Klein 1986). This indicates that, in C3 plants, a light-dependent process is required for the maintenance of functional rhythms of guard cell movements. However, in CAM species such as *Kalanchoë fedtschenkoi*, circadian rhythms of stomatal opening can persist for over one week in constant darkness, because the stomata respond to PEPC-induced circadian rhythms of C_i. Importantly, in both C3 and CAM plants, stomatal opening is phased to coincide with photosynthesis. In C3 and

C4 plants, this means that stomata are open in the day whereas in CAM plants the stomata are open during all, or part of the night, which corresponds to the phase of carbon fixation by PEPC (Webb 1998).

For optimal water use efficiency, rhythms of stomatal movement and photosynthesis are coordinated. When this coordination fails, there is a major physiological impact on the plant, as demonstrated by experiments investigating the advantage conferred by matching the endogenous clock period (τ) with the period of the exogenous light–dark cycle (T), i.e. circadian resonance (Pittendrigh and Bruce 1959; Ouyang et al. 1998; Dodd et al. 2005b). Wild-type, *toc1-1* and *ztl-1* plants were grown in either 10 h light/10 h dark (T=20), 12 h light/12 h dark (T=24) or 14 h light/14 h dark (T=28) cycles. Wild type were resonant with T=24, whereas *toc1-1* (τ=20.7 h; Somers et al. 1998) and *ztl-1* (τ=27.1–32.5 h; Somers et al. 2000) were resonant with T=20 and T=28 respectively. In all cases, when the oscillator was resonant with the environment, plants grew faster, fixed more carbon, and mature leaves contained more chlorophyll per unit area (Dodd et al. 2005b). Similar benefits were conferred by a functional clock in wild-type plants, compared with plants overexpressing *CCA1* (*CCA1*-ox) in T=24 (Dodd et al. 2005b).

In C3 and C4 plants, stomatal opening anticipates dawn in both LD and LL (Dodd et al. 2005b). In *CCA1*-ox plants, there are no circadian rhythms in stomatal conductance and there is also no pre-dawn increase in conductance when *CCA1*-ox plants are grown under light–dark cycles (see Fig. 8.2; Dodd et al. 2005b). Similarly, in wild-type plants stomatal conductance decreases in the afternoon whereas, in *CCA1*-ox plants, the stomata remain open until dark initiates closure. This indicates that the circadian clock is important in enabling stomata to anticipate changes in the fluctuating environment. It is particularly striking that the stomata of *CCA1*-ox continue to open until the light signal is removed. This demonstrates that, in unstressed wild-type plants, it is the clock, rather than the physiological or water status of the leaves, which arrests stomatal opening at midday and promotes the onset of closure in the afternoon (Fig. 8.2). One consequence of the circadian control of stomatal movements is that, in LD, wild-type plants use significantly less water than plants in which the circadian clock has been compromised by *CCA1*-ox. Thus, the clock may contribute to increased water use efficiency (Dodd et al. 2005b).

Misregulation of stomatal movements in *CCA1*-ox and plants in which the period of the clock is mismatched with that of the environment may contribute to reduced CO_2 assimilation (Dodd et al. 2005b). Incorrect phasing of stomatal movements with the environmental light cycle could result in a lag between light availability and CO_2 diffusion to the mesophyll. This could cause both reduced carbon fixation and light stress because the amount of reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced in the photosynthetic electron transport chain will exceed that which can be used in the Calvin cycle, so the excess light energy absorbed stimulates the

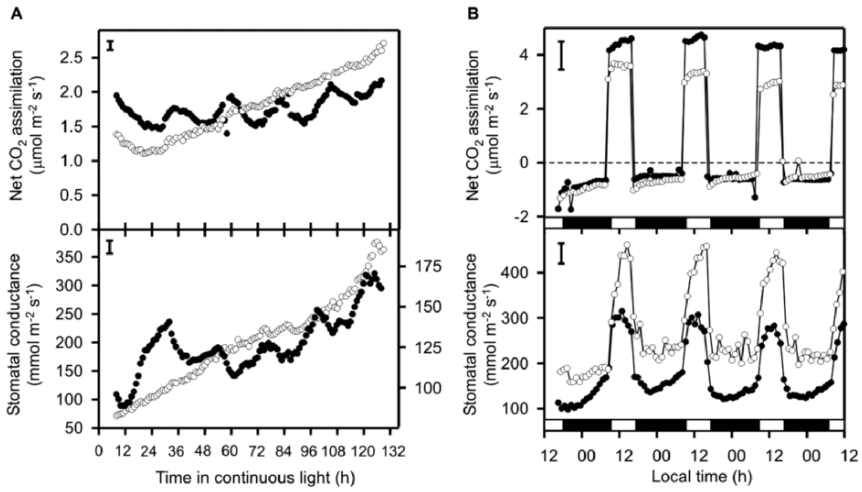

Fig. 8.2 Circadian and diurnal rhythms of photosynthesis and stomatal conductance in wild-type and CCA1-ox plants: infrared gas analysis of mature *Arabidopsis* rosettes. *Closed circles* represent wild-type plants (Col-0), *open circles* are the circadian arrythmic line CCA1-ox. **a** Rhythms in photosynthesis and stomatal conductance under continuous light. **b** Rhythms in photosynthesis and stomatal conductance under light–dark cycles (*open* and *closed bars* respectively). Data are means of six replicates (*vertical bars* largest standard errors of the mean). Reproduced from Dodd et al. (2005b), with permission from AAAS, Washington, DC

production of toxic ROS (Krieger-Liszkay 2005). There are many other circadian regulated processes that might also contribute to the reduction in carbon assimilation in plants that are not resonant with the environment. For example, much of the photosynthetic apparatus is circadian-regulated at the transcriptional level (Harmer et al. 2000; Schaffer et al. 2001; Edwards et al. 2006). While the amount of light-harvesting pigment and protein remains constant through the diurnal cycle, circadian transcription of photosynthesis-related genes may maintain steady-state levels by compensating for phase-specific turnover (Dodd et al. 2005b).

8.5 Structure of the Guard Cell Clock

Circadian rhythms of stomatal movements are probably controlled by a functionally independent circadian clock located within each guard cell. This is because the lack of plasmodesmatal connections with epidermal or mesophyll cells means that guard cells are sympastically isolated from the rest of the leaf, and so stomatal circadian rhythms are driven by a cell-autonomous oscillator (Gorton et al. 1989). The presence of a functional clock within each guard cell is demonstrated by the occurrence of rhythms of stomatal

movements in epidermis detached from the leaves for several days (Gorton et al. 1989). In epidermal peels, individual guard cells can become desynchronised from each other in LL, again indicating that guard cell oscillators are functionally isolated from those in other cells (Gorton et al. 1989). It is unknown whether endogenous mechanisms exist to synchronise the circadian rhythms of discrete stomata in intact leaves or if the clock is sufficiently robust to provide synchronisation between stomatal pores without the need for additional cell–cell signalling. The presence of an independent clock in the guard cell is consistent with current models of circadian organisation in plants, in which all cells (at least, in aboveground parts) contain a fully functioning circadian oscillator that can be entrained independently (Thain et al. 2002).

The presence of light perception and signalling networks in the guard cell presents the potential for entrainment of an oscillator by light–dark signals in the guard cell independently of other cell types. Does the guard cell circadian system therefore represent a cell-specific circadian oscillator with unique characteristics or does it have similarities with the consensus circadian oscillator described above? The evidence suggests that the guard cell circadian signalling network is organised in a manner similar to that regulating circadian rhythms of *CAB2::LUC* and leaf movements. As predicted by studies of *CAB2::LUC* and leaf movement rhythms, *toc1-1* shortens circadian rhythms of stomatal conductance (Somers et al. 1998), *ztl-1* lengthens rhythms of conductance (Dodd et al. 2004) and *CCA1*-ox results in arrhythmia of stomatal movements in LL (Dodd et al. 2005b). It is therefore likely that the circadian clock in the guard cell shares a common genetic structure with the clock found in other cell types, but this does not eliminate the potential for guard cell-specific clock factors that adapt the oscillator to the specialised role of guard cells.

As the clock confers considerable advantages to the plant (see above), it might be expected that there is coordination between the oscillators that control rhythms of stomatal movements and photosynthesis. However, this does not appear to be the case. The evidence for cell-autonomous rhythms in guard cells is described above and, in addition, it appears that the mesophyll oscillator is also cell-autonomous. Rhythms of *CAB2::LUC* luminescence, which emanate primarily from the mesophyll, can be entrained independently on the same leaf, and oppositely entrained rhythms persist on the same leaf in LL following entrainment. This strongly suggests that there is little communication between the cell-autonomous oscillators of the mesophyll (Thain et al. 2002). It also appears that there is little communication between the mesophyll oscillators and those of the stomata. Under unusual experimental conditions, it is possible to uncouple rhythms of photosynthesis and stomatal movements. If exposed to temperature cycles of 12 h at 28 °C and 12 h at 18 °C under LL, stomatal aperture in *V. faba* oscillates whereas carbon assimilation remains constant (Hennessey and Field 1991). Similarly, in the *ztl-1* mutant of *Arabidopsis*, rhythms of stomatal movements

and photosynthesis in LL have longer periods than in the wild type, but the period for rhythms of stomatal movement is 2.6 h longer than that for photosynthesis (Dodd et al. 2004). Thus, the periods for the two rhythms are uncoupled. Although the oscillators regulating photosynthesis and stomatal movements can become uncoupled, it is possible that there are physiological outputs of the rhythms that could act to reinforce appropriate behaviour at correct times in the cycle. For example, depletion of C_i in the sub-stomatal cavity by photosynthesis during the day in C3 and C4 plants will promote stomatal opening. In addition, there is considerable evidence that, in CAM plants, the nocturnal depletion of C_i by PEPC promotes stomatal opening, but this does not eliminate the possibility that the circadian clock in guard cells of CAM plants is modified for compatibility with CAM-specific temporal patterns of stomatal opening (Webb 2003). Finally, rhythmic changes in sucrose concentration due to rhythms of photosynthesis and metabolism may reinforce a number of rhythmic phenomena (Bläsing et al. 2005).

8.6 Mechanisms of Circadian Control of Guard Cell Physiology

The mechanisms through which the circadian clock is able to regulate complex physiological processes such as guard cell movements are currently unknown. With the exception of the photoperiodic flowering pathway (see Hayama and Coupland 2003, 2004 for reviews), very few genes downstream of the circadian clock have been assigned a role in linking the clock to physiological events.

The comparatively small number of verified output components of the circadian oscillator mean that it is not currently possible to determine if stomatal ion transporters and signalling pathways are directly controlled by the clock. Whole-plant circadian transcriptome analyses (Harmer et al. 2000; Schaffer et al. 2001, Edwards et al. 2006) have identified a few genes known to be involved in the control of stomatal movements and that are also regulated by the clock. Circadian-regulated transcripts include (1) *PHOTOTROPIN1*, which is involved in blue light perception for stomatal opening (Kinoshita et al. 2001), (2) *ARABIDOPSIS MULTIDRUG RESISTANCE-RELATED PROTEIN 5* (*AtMRP5*), an ABC transporter that is expressed in guard cells and is thought to regulate closure in response to ABA, and opening in the presence of auxin (Klein et al. 2003) and (3) *ARABIDOPSIS RESPIRATORY BURST OXIDASE HOMOLOGUE D* (*AtrbohD*), an NADPH oxidase subunit involved in ROS production downstream of ABA signalling in guard cells (Kwak et al. 2003). Of the ion transporters considered to be associated with stomatal movements, only *ARABIDOPSIS* K^+ *TRANSPORTER 2/3* (*AKT2/3*), which encodes a Ca^{2+}-sensitive inward-rectifying K^+ channel (Ivashikina et al. 2005), has been shown to be clock regulated at a transcriptional level. Compared

with mesophyll-associated transcripts, guard cell-specific components are likely to be underrepresented in these analyses, and it is unsurprising that few conclusions about guard cells can be drawn from whole-plant circadian datasets. There are currently comparatively few guard cell ion channels and signalling components described at a molecular level. Microarray analysis of guard cell protoplasts have identified 1,309 of 8,000 transcripts that are expressed in the guard cell, 64 of these being unique to the guard cell (Leonhardt et al. 2004). Until the molecular identities of all components responsible for the regulation of stomatal aperture have been identified, comparison of microarray datasets to identify circadian-regulated guard cell transcripts is of limited value. As many ion transporters are regulated post-translationally, transcriptomics may not be the best approach to probe the mechanisms involved in the regulation of stomatal aperture. At present, physiological approaches have provided the most insight into the possible mechanisms for regulation of circadian guard cell movements.

8.6.1 Calcium-Dependent Models for Circadian Stomatal Movements

$[Ca^{2+}]_{cyt}$ in leaves and cotyledons oscillates with a circadian period in LL, with an estimated trough concentration of 100–150 nM and a peak of 300–700 nM (Johnson et al. 1995; Love et al. 2004). $[Ca^{2+}]_{cyt}$ is a central regulator of stomatal movements, and the amplitude of circadian oscillations of $[Ca^{2+}]_{cyt}$ is sufficient to activate intracellular signal transduction pathways (Sanders et al. 2002). Therefore, a role for circadian oscillations of $[Ca^{2+}]_{cyt}$ in the circadian control of stomatal movements forms an attractive hypothesis (Webb 2003). Supporting evidence for a role for Ca^{2+}-based signalling pathways in the circadian regulation of stomata comes from studies of inhibition of phosphatidylinositol kinase activity, and of plants overexpressing phosphatidylinositol 3 phosphate (PtdIns3P) and phosphatidylinositol 4 phosphate (PtdIns4P)-binding proteins in the guard cell (Jung et al. 2002). These manipulations reduced stomatal opening in the light and dark during the early part of the photoperiod (Jung et al. 2002). The opening of stomata in either the light or dark during the early part of the subjective photoperiod may be partially due to the circadian clock. Phosphoinositide metabolism is important in guard cell Ca^{2+} signalling, and Jung et al. (2002) demonstrated that inhibition of PtdIns kinase activity inhibited ABA-induced increases in $[Ca^{2+}]_{cyt}$ in the guard cell. This study therefore provides one possible link between Ca^{2+} signalling mechanisms and the circadian regulation of stomata. However, this analysis did not include a true circadian time course, as events occurring in the first 24 h after entrainment are not considered true circadian rhythms. Thus, the results may reflect a perturbation of non-circadian cellular processes, rather than a circadian-specific effect. The technical difficulties in measuring circadian oscillations of $[Ca^{2+}]_{cyt}$ at a single-cell level currently limit our abilities to determine whether there are circadian

oscillations of $[Ca^{2+}]_{cyt}$ in the guard cell (Dodd et al. 2005a). Until we are able to determine circadian guard cell $[Ca^{2+}]_{cyt}$ dynamics, the role of circadian $[Ca^{2+}]_{cyt}$ oscillations in regulating stomatal physiology will remain unclear.

8.6.2 Calcium-Independent Models for Circadian Stomatal Movements

There may be alternative mechanisms regulating circadian stomatal movements, either in addition to, or instead of Ca^{2+}-dependent signalling pathways. One hypothesis, proposed by Tallman (2004), is that patterns of ABA biosynthesis and catabolism drive rhythms in stomatal aperture. In this model, diurnal conductance changes can be attributed to three phases of events. At dawn, photosynthetic electron transport is stimulated, resulting in the production of oxygen and NADPH. Since this occurs behind closed stomata, the oxygen concentration increases without a corresponding increase in C_i, which will result in photorespiration and limit Calvin cycle capacity. The NADPH produced by the photosynthetic electron transport chain is, therefore, in excess and can stimulate the activity of a cytochrome P450 that catalyses 8′ hydroxylation of ABA (Krochko et al. 1998; Kushiro et al. 2004), thereby reducing the guard cell cytosolic ABA pool. The ABA precursor violaxanthin (Xiong and Zhu 2003) is converted into zeaxanthin for photoprotection (Eskling et al. 1997), reducing the intracellular ABA concentration further. This relieves the repression of stomatal opening, so that solutes can accumulate. During the afternoon, ABA in the transpiration stream accumulates in the guard cell apoplast (Zhang and Outlaw 2001) and triggers ion efflux. H^+-sucrose antiporters enable sucrose accumulation in the cytosol to maintain turgor, and thus the guard cell remains open. However, if apoplastic ABA increases above a certain threshold – for example, when roots are subject to drought stress, or evaporation increases the effective concentration of ABA – then sucrose transporters are inhibited, so that there is net solute loss and the stomata close (Tallman 2004). At night, O_2 consumption in respiration and decreased concentrations of NADPH inhibit the ABA catabolism pathway. Violaxanthin levels increase, so that ABA synthesis in the cytosol is stimulated and the stomatal aperture decreases through ABA signalling pathways, as described above.

Evidence to support this model is currently incomplete; for example, it is not known how ABA synthesis occurs in guard cells, and the mechanisms through which ABA exerts different effects inside and outside of the cell are unclear. However, regulation of stomatal aperture through ABA concentration and distribution is an attractive idea, as the mechanisms through which ABA controls membrane transport processes are well characterised. In *Nicotiana tabacum*, ABA concentrations show a double peak during the day; there is a medium-sized peak in the late afternoon, followed by a larger peak at night (Nováková et al. 2005). The dual peak supports the model proposed, with the afternoon peak reflecting ABA delivery to leaf tissue via the

vasculature, and the night time peak relating to synthesis within the leaf. This analysis was conducted on whole-leaf tissue, however, so that it is not possible to say if this pattern would also be seen around the guard cells. Additionally, this model may not explain self-sustained free-running rhythms of stomatal opening occurring under constant conditions, since model perpetuation is at least partially dependent upon light–dark cycles.

There may be other mechanisms through which the circadian clock is able to regulate guard cell movements. It is likely that there will be many layers of control contributing to the rhythms in conductance that may or may not include the models described above. Until circadian rhythms are studied at the cellular level, the pathways linking the central oscillator to physiological outputs will remain elusive.

8.7 Circadian Regulation of Sensitivity of Environmental Signals ('Gating')

Guard cells are able to integrate a wide variety of signals that promote both opening and closure of stomata, and respond appropriately in order to balance CO_2 uptake against water loss (Hetherington and Woodward 2003). The circadian clock provides one mechanism through which the signals can be processed, as the response of stomata to extracellular stimuli can depend on the phase of the circadian cycle at which the stimulus is applied. This is known as circadian gating (Millar and Kay 1996), and implies that there are circadian regulated processes that favour one signalling pathway over another at given times of day. This means that the guard cells become more sensitive to closure signals at certain times of day, whilst opening becomes favoured at other times.

There is a circadian rhythm in stomatal responsiveness to light pulses, which shows that the light response of guard cells is gated by the clock (Gorton et al. 1993). *V. faba* seedlings exposed to a light regime of 2.5 h of darkness and 1.5 h of either blue or red light have greater responses to the light pulse in the middle of the subjective day than during subjective night (Gorton et al. 1993). This corresponds to the time of maximal stomatal conductance under continuous white light, and the time of the natural light intensity peak. Thus, the underlying circadian clock enables maximal light-induced stomatal opening at a time when the demand for CO_2 by the leaf is highest. The magnitude of the light response begins to increase before subjective dawn and continues to increase through the subjective day. This is surprising because, in *Arabidopsis*, the circadian clock promotes the onset of closure in the afternoon (Dodd et al. 2005b).

Circadian gating also reduces the effectiveness of closing signals such as ABA early in the subjective day (Correia et al. 1995), which enables high levels of CO_2 uptake in the morning before water becomes limiting in the late

afternoon. In addition, gating limits the ability of some signals, such as external K^+ and IAA, to open stomata during subjective night (Snaith and Mansfield 1985, 1986), again ensuring phase-appropriate response of the guard cells to external stimuli. Gating of cold-induced increases in $[Ca^{2+}]_{cyt}$ has been observed in *Arabidopsis* guard cells and seedlings, with maximal cold-induced increases in $[Ca^{2+}]_{cyt}$ during the day (Dodd et al. 2006). In this case, increased sensitivity to low temperatures during the day may be appropriate because low daytime temperatures reduce the capacity of the Calvin cycle, and this therefore requires the induction of photoprotective mechanisms to prevent ROS overproduction resulting from an excess of energy within the light-harvesting apparatus. An additional, tantalising hypothesis is that low temperatures are expected at night but, during the day, may indicate a change of season.

The mechanisms through which the circadian clock modulates responses to the environment are poorly understood at present. In whole seedlings, the acute response of *CAB2::LUC* to short pulses of white light is gated by the circadian clock in an ELF3-dependent manner (McWatters et al. 2000). Rhythmic expression of receptors and signalling components is likely to underlie many gating events, but this is another area in which more research is needed.

8.8 Conclusions

Regulation of both stomatal aperture and sensitivity of guard cells to environmental signals by the circadian clock is important for plant productivity and stress tolerance. Although the physiological processes underlying guard cell movements are well understood, how the clock regulates physiology at a molecular level is largely unknown. To further understand this interaction, there is a need for increased information on the molecular identities of ion transporters and signalling components that drive turgor changes following acute environmental stimuli. The gating of signalling pathway components by the clock will be a particularly interesting area of research in future, as this will provide us with greater understanding of how guard cells can integrate multiple conflicting signals and respond appropriately.

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Part 3
Rhythms, Clocks and Development

9 How Plants Identify the Season by Using a Circadian Clock

WOLFGANG ENGELMANN

This chapter is dedicated to my Doktorvater Erwin Bünning for the anniversary of his 100th birthday in 2006. He extensively studied photoperiodic events.

Abstract

Daylength as a function of the time of year (long days in the summer, short days in the fall, winter and spring) allows plants – and other organisms – to react photoperiodically in developmental steps and morphological features such as cyst formation in certain algae, succulence of stems and leaves, and the formation of storage organs and flowers. Bünning proposed in 1936 that the circadian clock of plants, with its 24-h cycling, is used in these photoperiodic reactions to measure daylength. Critical tests have corroborated this hypothesis. The functioning and molecular basis of the circadian clock of plants – especially of *Arabidopsis* – is presented and it is shown how this clock is entrained to the day. The photoperiodic timing of flower induction is more closely described.

9.1 Introduction and History

"Photoperiodism is a developmental control mechanism in plants and animals. The first phase of research work . . . has been characterized by studies in the biochemistry of the controlled processes like flower formation etc. This is fascinating, but not the real problem of photoperiodism. The second phase . . . is characterized by beginning studies in the control system itself. But these studies are still restricted to investigations on the type of clock used by organisms. The main problem for further research . . . should be how the responsiveness to light is controlled by the clock. This will be the third phase. But it will take some time to understand the electronics behind the organisms' switchboard."

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S. Mancuso and S. Shabala (Eds.)
Rhythms in Plants: Phenomenology, Mechanisms, and Adaptive Significance

This statement by Bünning (1969a), published almost 40 years ago, summarizes in wise foresight the way photoperiodic research has taken its way. In 2005, it was stated that "one of the fundamental problems facing biological scientists in the post-genome era is how to obtain, and test, models for the genetic networks that represent the regulatory 'wiring diagram' of a living cell" (Locke et al. 2005). Also, we still do not know well enough how the responsiveness to light is controlled by the clock and how 'the electronics behind the organisms switchboard' works. An enormous amount of knowledge and insight has accumulated during this period. New experimental methods and technical equipments have allowed us to dip into the molecular basis of biological clocks, used by organisms to tune their biochemistry and physiology to the daily and annual cycles of our planet. Modern theoretical approaches help to describe this complicated web of cellular interactions (Locke et al. 2005).

Seasonal changes are quite spectacular and evident especially at higher latitudes. They are the result of the 23° tilt of the rotating planet earth on its orbit around the sun. Climate, the environment and organisms are profoundly affected by it. The environmental factors temperature and daylength both correlate with the seasons but the latter is a more reliable indicator for us to establish the time of year. Based on a method to distinguish between days getting longer (spring) or shorter (fall), daylength would be a precise calendar, provided the timing device is independent of the environmental temperature.

An alternative calendar for an organism would be an internal annual clock. This is indeed realized in quite a number of organisms, also in plants (e.g. seed germination, Bünning 1951; water uptake, Spruyt and De Greef 1987; stomatal movement of bean seeds, Seidman and Riggan 1986). However, annual clocks must be synchronized, usually by the photoperiod. Without synchronization, after a few years an annual clock would no longer match the physical year because its period is not exactly 12 months.

Although biologists have long been aware of seasonal effects on life (and not only biologists, for that matter – in former times, it was common practice in Japan to evoke singing of 'Yogai' birds by offering artificial long days which induced the reproductive stage), the significance of the changing length of the light period during the course of the year was established relatively late in the 19th century (see Evans 1969b). Garner and Allard (1920) tried to identify why a giant mutant ('Maryland Mammoth') of tobacco flowered only during the winter, and found the short days to be responsible. They used the term 'photoperiodism' and demonstrated that flowering in soybean and other plants depends on daylength. Went (1959) discovered 'thermoperiodism', where the length of the high- or low-temperature period leads to reactions such as flower induction.

Many more observations and experiments on photoperiodism and thermoperiodism followed. At the same time, the biochemical and physiological

basis of these reactions were studied. Photoperiodic tuber formation in potatoes (Razumov 1931) and flowering in *Cosmos* (Garner 1948) are induced in the leaves. Sachs (1880) proposed an organ-forming substance in plants, and Knott (1934) extended this idea to the photoperiodic induction of flowering. Chailakhyan (1936) named this substance 'florigen' (later anthesin). Moshkov (1936) identified the leaves as the organs where this hormone is synthesized before it is transported to the apex. Pruning experiments confirmed this hypothesis (Lang and Melchers 1948). Short- and long-day plants seem to use the same flower hormone (Vince-Prue 1975, page 49ff). The role of the dark period, and the long-day effect of light breaks in the middle of the dark period were further milestones in studying photoperiodically controlled reactions and their mechanisms. For a recent historical essay which includes newer milestones in timing and photoperiodism, the reader is referred to McClung (2006).

Some important questions on photoperiodism we will deal with here are:

1. How is daylength measured?
2. What is the molecular basis of the photoperiodic timing?
3. Which photoreceptors are involved?
4. What are the mechanisms of photoperiodic reactions?

We will look at some examples of photoperiodic reactions in plants. The first is for a unicellular alga. The second example is the succulence of leaves in Crassulaceae. The third is tuber formation in potatoes. Finally, flower induction in short- and long-day plants is described. In the examples chosen, the level of complexity increases from a photoperiodic event in a single cell, to that in an organ such as the leaf, in underground stolons with remote perception of photoperiodic light in the leaves, and in the apex of stems, again with light perception in the leaves.

For the benefit of the reader, names of gene products are given here in alphabetical order for the abbreviations used in this chapter: AP1 (2), APETALA 1 (2); CAB, chlorophyll A/B-binding protein; CCA1, CIRCADIAN CLOCK-ASSOCIATED 1; CK2, casein kinase 2; CO, CONSTANS; CRY1 (2), CRYPTOCHROME 1 (2); DET1, DE-ETIOLATED 1; ELF3 (4), EARLY FLOWERING 3 (4); FD, flowering locus D; bZIP, transcription factor with basic domain and leucine zipper; FKF, FLAVIN-BINDING KELCH REPEAT F-BOX; FLC, FLOWERING LOCUS C; FRI, FRIGIDA; FT, FLOWERING LOCUS T; GA, gibberellic acid; GI, GIGANTEA; HD1, HEADING DATE 1; LFY, LEAFY; LHY, LATE ELONGATED HYPOCOTYL; LKP2, LOV KELCH PROTEIN 2; LOV, LIGHT OXYGEN VOLTAGE; LUX, LUX ARRHYTHMO; PER1 (2), PERIOD 1 (2); PHYA (B,C,D,E), PHYTOCHROME A (B,C,D,E); PPR5 (7,9), PSEUDO-RESPONSE REGULATOR 5 (7,9); SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1; SRR1, SENSITIVITY TO RED LIGHT REDUCED 1; TIC, TIME FOR COFFEE; TOC1, TIMING OF CAB EXPRESSION 1; ZTL, ZEITLUPE.

9.2 Examples for Photoperiodic Reactions

The unicellular dinoflagellate *Lingulodinium polyedra* is found at the surface of the oceans during the warmer part of the year. In the fall, the cells sink to the bottom of the sea, get rid of their shells and flagellae, and form a cyst. This step is photoperiodically controlled (Balzer and Hardeland 1991). Temperatures above 16°C prevent cyst formation. The photoreceptor(s) is (are) not yet known. Circadian rhythms in *Lingulodinium* are synchronized by blue and red light (Roenneberg and Hastings 1988). Photoperiodic reactions are known also in other algae (conchiospores of *Ulva*, monospores in *Porphyra*; see overview by Lüning 1980).

Succulence of stems and leaves is also often under photoperiodic control. Under short-day conditions, *Kalanchoe blossfeldiana* forms small, rigid, succulent leaves. Under long-day conditions, the leaves are larger, thinner and flexible (Denffer 1941). Water uptake and subsequent enlargement of the cells perpendicular to the leaf blade lead to succulence (Harder 1948). Metaplasin was proposed to induce succulence, because grafting of succulent leaves to plants under long-day conditions induces succulence in these plants (Harder and Witsch 1940). The flower hormone florigen is, however, said to be different from metaplasin, as chloroform inhibits flower induction in short-day regimes but has no effect on the thickening of the leaves (Harder and Gall 1945). Furthermore, some Crassulaceae are known to be succulent in short-day regimes but to flower under long-day conditions. The photoreceptors for the photoperiodic control of succulence of *Kalanchoe* leaves are unknown – Wallrabe (1942) found red and blue light to be effective whereas green light was not.

Numerous storage organs of plants are induced photoperiodically (Ewing and Struik 1998). Wild type and varieties of potatoes from South America form tubers on their stolons (underground stems) under short-day conditions (Hackbarth 1935). Red is the light effective and is perceived by phytochrome B in the leaves (Jackson and Thomas 1998). If the critical dark period is surpassed, tuber-inducing substances (tuberonic acid, jasmonic acid; Koda et al. 1988) are produced. At the same time, a long-day tuber-inhibiting substance (gibberellic acid; Tizio 1971) looses its effect under short-day conditions. The activity of enzymes in the pathway of tuberonic acid and jasmonic acid biosynthesis and in the gibberellic acid pathway is photoperiodically controlled (lipoxygenase and gibberelline-synthetase respectively; Gilmour et al. 1986). Tuberonic acid and jasmonic acid inhibit the growth of the stolons in the longitudinal direction and induce thickening. Furthermore, starch is stored in the cells.

Flowering is the most frequently studied photoperiodic reaction. It can be induced by long or by short days, depending on the species or variety. The length of the light period or dark period is not important here, rather whether a critical length has at least been reached or not. During the development of a plant, several photoperiodic switches can become effective which might

belong to different photoperiodic types (long day, short day). *Zea mays* is a good example (for details, see Vince-Prue 1975; Thomas and Vince-Prue 1997). Usually, the plant needs a number of inducing photoperiods in order to flower. In some plants, however, only one day is sufficient. *Pharbitis nil* (Chois) cv *violet* and *Chenopodium rubrum* (L.) are examples for short-day plants, and *Lolium perenne* for a long-day plant (in Evans 1969a). Since the photoperiodic treatment also leads rapidly to the photoperiodic reaction, these plants are preferred for photoperiodic experiments. Other photoperiodic effects are known in plants, such as root formation, leaf abscission, bud formation and bud dormancy of trees and shrubs, stem elongation, vegetative growth, cambium activity, tissue differentiation, cold resistance, fertility as well as sex determination and expression (for lack of space, we refer to Vince-Prue 1975, and Thomas and Vince-Prue 1997).

9.3 Bünning Hypothesis and Critical Tests

Photoperiodic regulation of a biological process simply means that one partial process is linked with a time measurement. Organisms have an inherited timescale at their disposal. Day by day, throughout the year, they compare this scale with the actual length of the day or night. As soon as the actual length of the day or night is greater (or smaller, as the case may be) than this inherited scale, a change in physiological make-up occurs. This is what we call a 'photoperiodic reaction', and the inherited timescale is known as the 'critical day length' (Bünning 1960).

Given a changing photoperiod throughout the year, an engineer would probably use a kind of hourglass or stopwatch to construct a timer for measuring the length of the light period or the dark period. If a certain time span, corresponding to the length of the day at which something should be done, has passed, the stopwatch rings or the sand of the hourglass has accumulated in the lower part. Now, action (in organisms: photoperiodic reactions) could be taken.

However, that is usually not the way organisms measure daylength. Rather, they use a pendulum-like clock, as proposed by Bünning (1936). According to this Bünning hypothesis, as it was later called, light has two functions: (1) it synchronizes the circadian clock and (2) it induces the photoperiodic reaction, or not, depending on the photoperiodic constellation of the season (long days or short days) and the photoperiodic situation of the organism (for instance, long-day plant or short-day plant; see Fig. 9.1).

In both cases, the circadian clock plays an important role:

1. A light pulse during the subjective day (i.e. the portion of the circadian cycle which would correspond to the light period, if an organism is kept under continuous darkness or continuous light) does not shift the rhythm

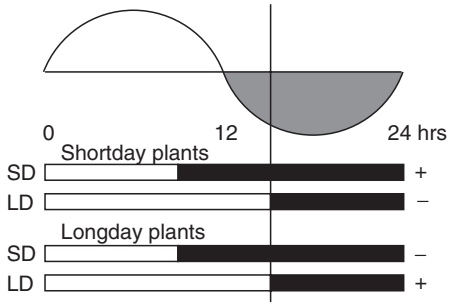

Fig. 9.1 The Bünning hypothesis explains how a circadian clock measures daylength. *Above* The curve shows the photophilic phase (*nonshaded area*) and the skotophilic phase (*shaded area*). *Below* A short-day plant would be induced to flower (+) in short days (*SD*) with dark periods (*shaded*) exceeding a critical length but would not flower (–) if light falls during the skotophilic phase in long days (*LD*). Long-day plants would react in reverse to short-day plants (*bottom*)

because, at that time, the organism is normally subjected to light. A light pulse during the subjective night, however, shifts the rhythm. Light pulses shortly before midnight act strongly by delaying the rhythm whereas light pulses after midnight advance the rhythm. This can be visualized by a phase-response curve. A light pulse given at exactly the subjective midnight dampens the rhythm, if it has the appropriate strength. I mention this because it could also be used to test the Bünning hypothesis. If the clock measures daylength, as claimed by Bünning, then the measurement should no longer work if the clock is stopped from running. This has been done with *Pharbitis nil* (Engelmann, unpublished data).

2. Depending on daylength, the internal oscillation with its two alternating phases (Bünning called these 'photophilic', light-loving, and 'skotophilic', dark-loving) interferes with the external light–dark cycle in different ways (see Fig. 9.1).

One important point is how the circadian clock for photoperiodic timing is synchronized by the light–dark cycle. In the case of bean plants, Bünning assumed that the onset of light is responsible for this. However, the beginning of the dark period could also be responsible, as has been shown in short-day plants such as *Pharbitis* (Lumsden and Millar 1998). This point is discussed further below.

In the internal coincidence model, a light-on rhythm and a light-off rhythm (or a morning and an evening oscillator; Daan et al. 2001) are set in motion by a change from darkness to light and from light to darkness respectively. Both rhythms interfere with each other differently, depending on the length of the light period. This could be responsible for the photoperiodic reaction, as suggested by experiments in *Kalanchoe blossfeldiana* (Engelmann 1960). This model accommodates the varying daylengths during the year,

which can not be done with a model using a fixed time delay starting from only one phase, such as dawn (Rand et al. 2004).

However, it is not necessary to consider here two rhythms (M- and E-oscillator). Using the feedback model of Johnsson and Karlsson (1972) and an appropriate approach to enter light into the feedback loop, photoperiodic reactions of flowering in *Chenopodium rubrum* could be simulated by using only one oscillator, rather than two (Bollig et al. 1976). Furthermore, Rand et al. (2004) point out that one oscillator with two or more loops, one of which is locked to dawn whereas the other is locked to dusk, can cope with the seasonal change of daylength (see the models for photoperiodic reactions in mammals and the involvement of clock genes *per1* and *per2*, *cry1* and *cry2*; references in Rand et al. 2004).

The main points of the Bünning hypothesis are that a circadian clock is used for measuring the daylength in photoperiodic reactions, that this clock is synchronized by the light–dark cycle and that the photoperiodic reaction occurs if the cycling of the clock coincides with the proper light or darkness situation in the environment. A number of experiments have been designed and performed to test the hypothesis.

Thus, varieties of soybeans from different latitudes possess different critical daylengths for photoperiodic flower induction. Moreover, the more southern varieties fold their leaves earlier in the evening as compared to the more northern varieties, indicating differences in the clocks (Bünning 1979). Daylength is more precisely measured in plants which are able to move their leaves either via pulvini at the base of the blade and the petiole (such as in Leguminosae) or via alternating growth of the upper and lower side of the leaves (such as in various Solanaceae and many other plants): the rapid change in light intensity at dawn and dusk is further intensified by moving the leaves from a vertical night position to a horizontal day position (Bünning and Moser 1969). Furthermore, a possible adverse effect of moonlight during the night is prevented, which could otherwise interfere with the photoperiodic reaction (Bünning 1969b).

Variation of the dark period in short-day plants was used as another test of the hypothesis. If the hourglass hypothesis were correct, then groups of plants receiving a fixed light period and varying lengths of dark periods should flower once the critical dark period is surpassed. It turned out, however, that the amount of flowering depended rhythmically on the length of the dark period (cf. experiments on soybeans by Blaney and Hamner 1957, and Fig. 9.2).

- Light breaks in a long dark period: likewise, a short light break given to different groups of plants in a long dark period led to a rhythmic response (see Melchers 1956 and references therein, and Fig. 9.2).
- Slowing the clock affects the critical daylength: if daylength were measured by a circadian clock in photoperiodic flower induction, then the critical daylength should change if the speed of the clock is changed. This was

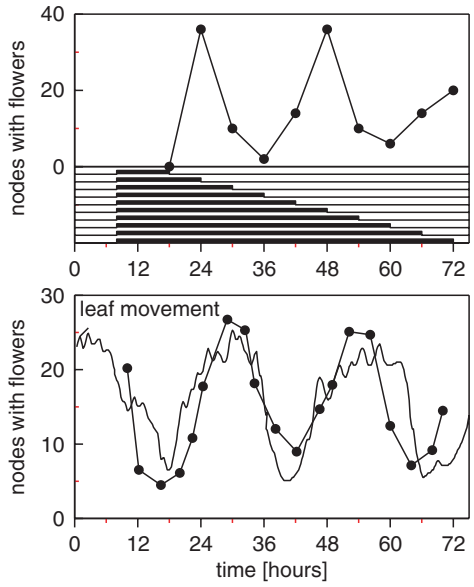

Fig. 9.2 Tests of the Bünning hypothesis. *Above* The photoperiodic reaction of soybeans to dark periods of varying lengths (see *shaded bars*) is rhythmic (*curve* shows the number of nodes with flowers). *Below* The photoperiodic reaction of soybeans to light breaks in a long dark period (64 h) is rhythmic and the leaf movement rhythm has the same time course under the same lighting conditions (i.e. 8:64 h LD). After Bünning (1954), by kind permission of Springer, Heidelberg as copyright owner

shown to be the case by slowing down the clock with heavy water D_2O (Brenner and Engelmann 1973) or with lithium ions (Engelmann et al. 1976).

- Mutants with defects in photoperiodic control of flowering: as a further test of the Bünning hypothesis, mutants with defective photoperiodic flowering, such as *elf3*, *lhy*, *gi* and *elf4*, also showed aberrant circadian rhythms (references in Yanovsky and Kay 2003).
- Mutants with circadian defects: conversely, it has been demonstrated that the photoperiodic sensitivity of mutants selected for defects of the circadian clock, such as *toc1* and *ztl*, has changed. This is particularly true for *toc1-1*, an allele to *toc1* with a 21-h period length. In contrast to the other cases of mutants mentioned above, in which photomorphogenetic changes are also observed which could indicate changes in light signalling (rather than circadian signalling), *toc1-1* lacks light-dependent morphological phenotypes (references in Yanovsky and Kay 2003). Under a 24-h light–dark cycle, this mutant flowers early independently of daylength. However, under a 21-h light–dark cycle, which corresponds to the speed of its internal clock, flowering is induced under long-day conditions only. This is a strong argument in favour of the Bünning hypothesis.

9.4 The Circadian Clock and its Entrainment to the Day

Most organisms possess circadian clocks to time many of their biochemical and physiological processes. At least 6% of the RNA transcripts of the *Arabidopsis* genome, i.e. more than 1,000 genes, is rhythmically expressed under circadian clock control (Harmer et al. 2000). The adaptive advantages of possessing a circadian clock have been experimentally tested and confirmed by Green et al. (2002) and Michael et al. (2003). The period of these clocks is close to 24 h and remains constant over a range of temperatures (mechanism discussed by Gould et al. 2006). The clocks are synchronized by environmental time cues such as the light–dark cycle which, via photoreceptors, signal light to the clock. However, temperature cycles (higher temperature during the day, lower temperature during the night) do also synchronize the circadian clock.

The underlying clock mechanisms are quite conserved among animals but less so in fungi and cyanobacteria. As in animals and fungi, the oscillators in plants are largely considered to consist of transcriptional/translational negative feedback loops (see, however, a paper by Lakin-Thomas 2006 as a caveat) but the clock components of plants differ from those of animals and fungi. The molecular composition of the *Arabidopsis* clock, as proposed first by Alabadi et al. (2001), has become quite complex in the meantime (for reviews, see Salome and McClung 2005, and Màs 2005), and several groups in various countries are currently at work on it (see the Internet sites given on dartmouth.edu/~rmcclung/links.html). As often done in cases where we are dealing with complex and interwoven networks, model building is an efficient way of handling this (e.g. Rand et al. 2004, Locke et al. 2005, and references therein). In a simplified version, three negative feedback loops interlock with the two transcription factors CCA1 and LHY2, as shown and described in Fig. 9.3. In addition to this transcriptional regulation, post-transcriptional events are essential for the clock to work (Daniel et al. 2004).

Of the various photoreceptors (consisting of a light-absorbing chromophore bound to an apo-protein, an effector domain important for dimerization and protein–protein interactions; absorbed light changes the conformation or chemical structure of the receptor apo-protein, and this is signalled downstream) of plants, at least PHYB and CRY2 are used for synchronizing the circadian clock (most work has been done in *Arabidopsis thaliana*; cf. reviews by Devlin and Kay 2001, and Millar 2004). PHYB is a red-absorbing photoreceptor and it interacts with the blue light-absorbing CRY2 (Devlin and Kay 2000; Màs et al. 2000; Jarillo et al. 2001). In turn, the clock affects the photoreceptors which are synchronizing it via feedback, a feature termed 'Zeitnehmer' (German for time taker, in contrast to 'Zeitgeber', time giver or time cue). The light input pathways to the clock, indicated in Fig. 9.3, are not yet well understood (Fankhauser and Staiger 2002; Nagy and Schäfer 2002; Viczian et al. 2005).

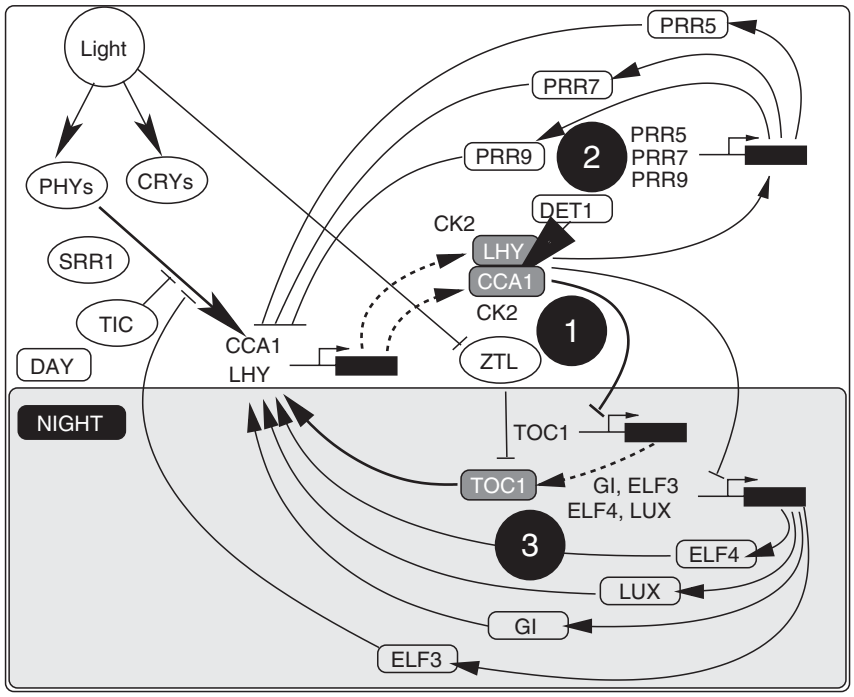

Fig. 9.3 Molecular composition of the *Arabidopsis* clock. Three negative feedback loops (*1*, *2* and *3* in *shaded circles*) interlock with the two transcription factors CCA1 and LHY. In loop 1, CCA1 (cf. definitions of abbreviations in Sect. 9.1) and LHY bind to the promoters of *toc1* to inhibit their expression (⊤). *cca1* and *lhy* are induced by light (via phytochromes PHYs) and expressed rhythmically, with mRNA and protein abundance peaking at dawn. TOC1-mRNA oscillates and peaks at dusk, and its protein regulates CCA1 and LHY positively (→). In feedback loop 2, CCA1 and LHY regulate three *toc1* relatives, *prr5*, *prr7* and *prr9* negatively. PRR5, PRR7 and PRR9 repress *toc1* mutually. PRR9 is rhythmically expressed at subjective noon and is induced by red light via phytochromes. In succession of 2-h intervals, the peaks of PRR7, PRR5 and PRR3 rhythms follow. The function of the PRRs is not yet understood. In feedback loop 3, CCA1 and LHY negatively regulate *gi*, *lux*, *elf4* and *elf3*. ELF3 serves as a 'Zeitnehmer' by rhythmically inhibiting the PHY-inputs to the clock system. In addition to the transcriptional regulation, post-transcriptional events are essential to the functioning of the clock. For instance, CK2 (casein kinase II) phosphorylates CCA1 and leads, via proteasomes, to degradation. The LOV domain of *fkf* (not shown) is photoactive, and FKF is restricted to photoperiodism. ZTL and LKP2 (not shown) affect the clock. ZTL recruits TOC1 for proteasomal degradation especially during the day. At dusk, at its peak value, ZTL is more stable. LHY (not shown) is rapidly degraded via proteasomes. The *upper part* of the figure indicates high activities during the day, the *shaded lower part* high activities during the night. After McClung (2006), by kind permission of the author and the American Association of Plant Biologists, Rockville, MD as copyright owner

Additional photoreceptors with chromophores are ZTL which, after absorbing light, mark protein components of the clock such as TOC1 for proteosomal degradation (Màs et al. 2003). This represses PHYB/CRY2 signalling to the clock by physically interacting with PHYB. Still, there seem to be additional routes where proteins without chromophores, such as TIC, ELF3 and SRR1, interact directly with photoreceptors. Whereas ELF3 functions as a light gate at night, TIC gates the clock at daytime. The double mutant *tic;elf* is arrhythmic (Hall et al. 2003). The different phytochrome and cryptochrome photoreceptors interact with each other, and show complex responses at different expression levels, localizations, and accessibility to signalling intermediates. This communication between photoreceptors and transduction pathways enables a simultaneous response to two or more environmental parameters (Ahmad 1999; Halliday and Whitelam 2003).

9.5 Seasonal Timing of Flower Induction

In order to flower and produce seeds at the right time, plants use several control systems to evoke the transition from the vegetative to the reproductive stage of the apex (see Fig. 9.4). One of these control systems depends on the developmental stage: The plant has to go through a juvenile phase and to reach a certain age before flowering is induced. This is the autonomous pathway of flowering. Another part of the control system perceives environmental signals such as daylength, temperature and humidity of the soil. If this environment-reactive pathway reports the right conditions, flowering is induced. Whether the autonomous or the environment-reactive pathway prevails depends on the plants in question, and varies strongly (Aukerman and Amasino 1996).

Recently, the molecular mechanism of flower induction has been clarified a great deal by using forward and reverse genetics, genomic technologies (Wellmer and Riechmann 2005) and new techniques such as microarray analysis (Yamada et al. 2003). In the following, the photoperiodic induction in the environment-reactive pathway will be treated. It is at the same time the best studied photoperiodically controlled event in plants.

Figure 9.3 shows a simplified scheme of the current view for the long-day plant *Arabidopsis thaliana*. The photoperiodically active light is perceived by photoreceptors in the leaves, and there perhaps in the mesophyll and in the epidermis, as has been shown, for instance, in *Kalanchoe blossfediana* by removing the upper and lower epidermis respectively, which reduced flowering dramatically (Schwabe 1968), in *Solanum* by using periclinal chimeras, and by photoperiodically illuminating leaves of *Chenopodium amaranticolor*, *Kalanchoe* and *Perilla ocymoides* either through the upper or lower epidermis (Bünning and Moser 1966; Schwabe 1968; Mayer et al. 1973), leading to different responses. The epidermis of several plants can show specialized

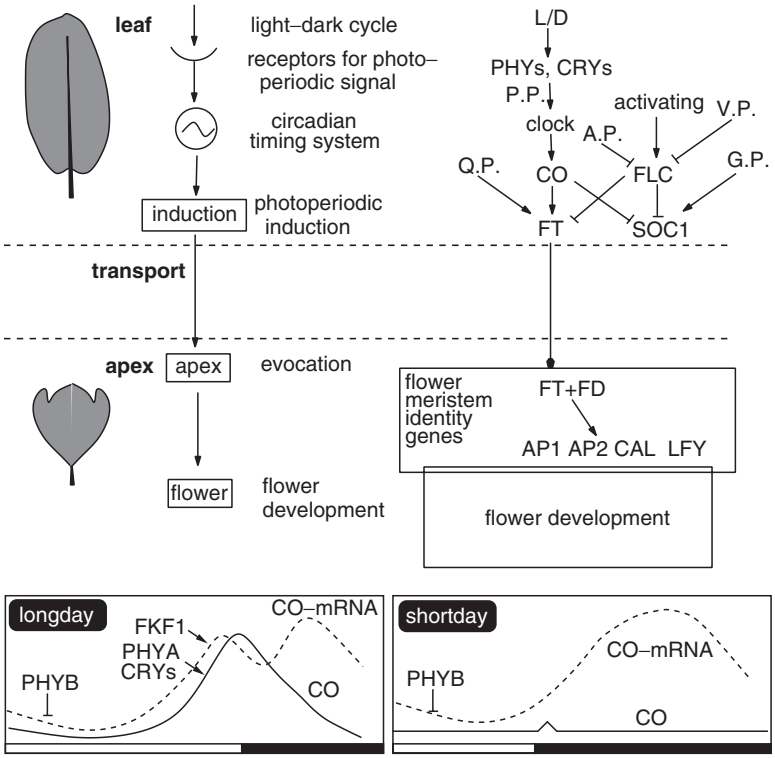

Fig. 9.4 Scheme of the flower induction of a long-day plant. The photoperiod (*L/D*) is perceived by photoreceptors in the leaves (PHYs and CRYs; cf. definitions of abbreviations in Sect. 9.1). Under long-day conditions, FT is produced and transported to the apex. There it combines with FD and activates flower meristem identity genes *ap1*, *ap2*, *cal* and *lfy*, thereby initiating flower development. There are other pathways, besides the photoperiodic one (*P.P.*), which control flowering, such as an autonomous pathway (*A.P.*, which eventually leads to flowering even under non-inductive photoperiodic conditions), a light quality pathway (*Q.P.*, which accelerates flowering under shading conditions), a vernalization pathway (*V.P.*, a treatment where low temperature is necessary for flowering to occur) and a gibberellic acid pathway (*G.P.*, in which GA will induce flowering). The A.P. and V.P. pathways inhibit *flc*, thereby removing the blockade of FT production by FLC, or activate *ft* (Q.P. via PFT1, which is not shown) or (G.P.) activate *soc1* and the flower meristem identity genes directly. There exists also an activation of FLC (*activating*). The photoperiodic reaction is due to rhythmic control of co-expression (*left curve at bottom*, CO-mRNA) by the circadian clock, FKF1 protein functioning as a photoreceptor, and the fact that CO is stabilized by CRYs and PHYA under light conditions but is unstable in darkness. PHYB antagonizes the activity of CRYs and PHYA especially in the morning. It takes some time in light (a long light period, *left bottom curves*) until sufficient CO has accumulated to induce FT expression, which is necessary for flower induction. In short days, there is not enough time under light to produce the CO protein (*right bottom curves*). After Màs (2005), by kind permission of the author and the International Journal of Developmental Biology, Bilbao as copyright owner

anatomical features which enable sufficient light absorption during twilight (Haberlandt 1905). Phytochromes are the most important photoreceptors for flower induction, e.g. in peas (Weller et al. 1997). In Cruciferae, photoreceptors for blue light are also involved (Guo et al. 1998). There are opposing roles played by PHYA and PHYB in flowering (by differentially regulating FT expression, see below). Signalling occurs via a network with molecular interactions. This differs under short- and long-day conditions, and the environmental temperature changes the importance of the different photoreceptors (Halliday and Whitelam 2003).

In the leaf, daylength is measured by a circadian clock (see Sects. 9.3 and 9.4): if the critical dark period has been surpassed in short-day plants or if this falls short in long-day plants, then a substance is produced by the cells in the leaf for flower induction. Daylength measurement has to be accurate even at varying environmental temperatures. Thus, the critical daylength is, in the case of *Kalanchoe blossfeldiana*, the same at 28 and 18 °C. However, the number of induced flowers is quite different. At the higher temperature, 40 times as many flowers are formed as at the lower temperature. This temperature compensation of the critical daylength is found not only at flower induction but also during other photoperiodic reactions.

CO (CONSTANS) plays a central role (Putterill et al. 1995): it mediates between the environment, the clock and the initiation of flowering (Hayama and Coupland 2003). CO-mRNA oscillates in a circadian manner. Under long-day conditions, the peak is at the end of day and during the night and, under short-day conditions, during the night (see curves in Fig. 9.4). Seeing that, in a long-day plant such as *Arabidopsis thaliana*, CO is stable only in the light (CO accumulates in blue light and far red light but not in red light; PHYA and CRYs stabilize CO at the end of day) and is degraded by proteosomes in darkness (PHYB promotes CO degradation during the dark period and leads to low concentrations of CO in the morning; Hayama and Coupland 2004), it will be present in long days but not in short days. The increase in the amount of CO during short days in the early part of the dark period is very minor and not sufficient to induce the synthesis of FT by *ft*, a target gene of CO (Valverde et al. 2004). Under long-day conditions, by contrast, sufficient CO will accumulate and lead to FT-mRNA production. This is essentially what the external coincidence model requires (Suarez-Lopez et al. 2001).

As mentioned above, a mutant *toc1-1* exhibits a short free-run period length of 21 h. It demonstrates nicely that this coincidence model is valid (Yanovsky and Kay 2003): it expresses CO in both short and long days, and flowers early without any photoperiodic control. If the mutant is kept under a 21-h day, however, it reacts photoperiodically again: a long day (on a 21-h basis!) induces flowering, a short day does not. Thus, the coincidence of CO and the correct photoperiod (long day in long-day plants) is a key event in the photoperiodic control of flowering time (Roden et al. 2002; Yanovsky and Kay 2003).

FT-mRNA is transported from the leaves to the place of action in the apex. FT is a small protein and might also be transported to the apex (Huang et al. 2005). In any case, FT (or the product of another gene which is induced by FT) combines with FD, which is present at the apex but inactive without FT. The FT–FD complex initiates reproductive development (flower evocation; Abe et al. 2005; Wigge et al. 2005). Flower meristem identity genes such as *ap1*, *ap2*, *cal* and *lfy* are activated and flowers are induced according to the ABC (DE) model (Parcy 2005).

The CO–FT interaction is conserved among plants (Griffiths et al. 2003; Izawa et al. 2003). The photoperiodic responses are conferred by the same genetic pathway in the long-day *Arabidopsis thaliana* and the short-day rice *Oryza sativa*. Nevertheless, the functions differ (Hayama and Coupland 2003). In short-day plants, CO is reported to inhibit FT. Long dark periods would then promote FT expression, because CO activity is low. At the biochemical level, it is not yet known how the switch works which leads to flowering in long-day plants under long-day conditions and in short-day plants under short-day conditions (Cremer and Coupland 2003).

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10 Rhythmic Stem Extension Growth and Leaf Movements as Markers of Plant Behaviour: the Integral Output from Endogenous and Environmental Signals

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Dedicated to the 80th anniversary of Bruce G. Cumming

Abstract

With the model systems *Chenopodium rubrum* (short-day plant) and *Chenopodium murale* (long-day plant), growth and behaviour have been studied in response to photo- and thermoperiod. With time-lapse photography, rhythmic integration of the plant as a whole could be monitored. Upon photoperiodic flower initiation, rhythmic stem extension rate (SER) and leaf movement (LM) change their phase relationship in a specific way. Flower induction correlates to a threshold value for the ratio between integral growth during the dark time span and integral growth during the light time span. This precise output displayed in the growth pattern of the plant is therefore an accurate reflection of all available environmental inputs.

Analysis of flower induction in *Chenopodium* spp. showed that, 2 h after the end of the critical dark period, the patterns of cytoplasmic pH and Ca^{2+} change at the shoot apical meristem (SAM), possibly indicating the arrival of the flower-inductive signal. Changes in LEAFY and aquaporin expression can also be recorded during this phase. The perception of a flower-inducing dark period probably leads to a change in electrochemical, hydraulic signalling between the leaves and SAM, thereby determining polarity in the whole plant and paving the way for "florigen", the flower-inducing hormone postulated in 1936 but still undiscovered.

A rhythmic integration over the whole plant, as seen for SER and LM, most likely involves modulation of turgor pressure via stretch-activated ion channels and concomitant changes in membrane potential, making the plant a hydro-electrochemical signal transducer. Regulation of hydraulics and electrochemistry, two coupled physicochemical processes, was an achievement of early evolution as well as metabolic circadian regulation of transcriptional

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Rhythms in Plants: Phenomenology, Mechanisms, and Adaptive Significance

translational control loops (TTCL). Circadian rhythms (CRs) in energy metabolism are gating inputs and outputs to the TTCL, resulting in a CR of protein synthesis and turnover. Evolution of latitudinal ecotypes with different CR period lengths will depend on specific proteins, as is evident from early crossing experiments. The control of the ionic composition of the cell is crucial for survival and requires energy to maintain a resting potential of the plasma membrane. This, in turn, enables the generation of action potentials and, hence, a fast systemic communication between plant organs.

10.1 Introduction

10.1.1 Life is Rhythmic

Rhythmicity is one of the characteristics of life which expresses itself at all levels of organisation, from unicellular systems to man. Rhythmic phenomena in the physiology, development and behaviour of all living systems show period lengths ranging from fractions of a second to hourly, daily and even annual cycles (Cumming and Wagner 1968; Lloyd and Rossi 1992; see also Chap. 13 by Lloyd, this book).

The most conspicuous rhythm is the so-called diurnal rhythm. The diurnal rhythm is based on an endogenous oscillation of metabolic activity with a period length of exactly 24 h when organisms are synchronized by the daily light–dark cycle of the earth. Under constant conditions, however, its period length is only approximately 24 h, i.e. circadian. Even in the Arctic summer there is a synchronizing, daily cycle of light intensity and light quality. In contrast to biological rhythms showing other frequencies, circadian rhythms are temperature compensated and almost totally unsusceptible to chemical manipulation. It is this stability, or homeo-dynamics, of period length which qualifies the circadian rhythm as a precise physiological timer, this being the essence of Bünning's (1973, 1977) theory of the physiological clock (see also Chap. 9 by Engelmann, this book).

In their natural environment, plants develop under daily cycles of light–dark and high–low temperatures. The change of seasons is associated with characteristic fluctuations in day length and in the phase relationship between the photo- and thermoperiod. In spring and winter, light and temperature change in parallel in a so-called radiation climate. In summer and early fall, the coldest point in the day is around sunrise. At sunset, however, the light "goes off" while the temperature slowly decreases until sunrise. Changes in temperature and light intensity are therefore not synchronous at sunset. Plants have adapted their development to such environmental conditions by the evolution of photo- and thermoperiodic responses, e.g. thermoperiodic control of phototropic responsiveness (Schwall et al. 1985). The basis of such responses are endogenous changes in sensitivity to environmental light- and temperature signals (Wagner et al. 1983).

In laboratory experiments, however, constant environmental conditions are often used for analytical and practical reasons, e.g. to demonstrate the existence of a circadian rhythm with a temperature-compensated period length different from 24 h. If, under constant environmental conditions, an oscillation with a periodicity of exactly 24 h is observed, then subtle geophysical synchronizers, so-called zeitgebers, might be involved (Bünning 1977).

In sensitive plants, constant conditions can lead to degradation of the photosynthetic apparatus and, when applied for a longer period of time, to the death of the plant. This is probably the result of internal desynchronization of metabolic cycles (Arthur et al. 1930). In other words, internal biological rhythms of the plant require external rhythmicity for synchronization and, hence, for survival. Pathologic effects of constant conditions can be reversed by applying either photo- or thermoperiodic 12:12 h cycles, even at very low amplitudes. Slight variations of parameters which might act as external zeitgebers are apparently sufficient for resynchronization or rephasing of metabolic activities (Went 1944, 1957; Highkin and Hanson 1954; Hillman 1956; Highkin 1960). Internal desynchronization might be involved in the aging and senescence of plants and animals. Even at the equator, where no seasonal change in day length occurs, there is still a seasonal rhythm in dawn and dusk signals with respect to their positioning in the daily 24-h cycle. This very fine seasonal change synchronizes low-density populations of tropical trees, thereby securing cross-pollination for flowering (Borchert et al. 2005).

An adaptation to stable differences in a seasonal change in photoperiod is indicated by so-called latitudinal ecotypes, as isolated for *Chenopodium rubrum* L. at various locations in North America (Fig. 10.1; Table 10.1). The three ecotypes differ in the period length of their genetically fixed endogenous rhythm and have different critical photoperiods for flower initiation. The circadian system (the clock's periodicity) is genetically determined, as has been demonstrated by crossings affecting the period length of endogenous leaf movements (Bünning 1935, 1977). The genetic determination of period length provides the temporal frame for physiological and behavioural patterns which are necessary for adaptations of organisms and populations to environmental constraints, as evident for three latitudinal ecotypes of *C. rubrum* (Table 10.1; cf. Cumming 1967; Tsuschiya and Ishiguri 1981).

10.1.2 Rhythm Research: Metabolic and Genetic Determination of Rhythmic Behaviour

Research into so-called sleep movements of leaves supplied the basic kinetic information for Bünning, who developed the theory of the physiological clock (Bünning 1973, 1977). Here, he characterised and described a circadian (~24 h) temperature-compensated oscillation as the cellular time-measuring principle of eukaryotic and, as demonstrated more recently by Golden et al. (1997), of prokaryotic systems. Bünning's work paved the way for more

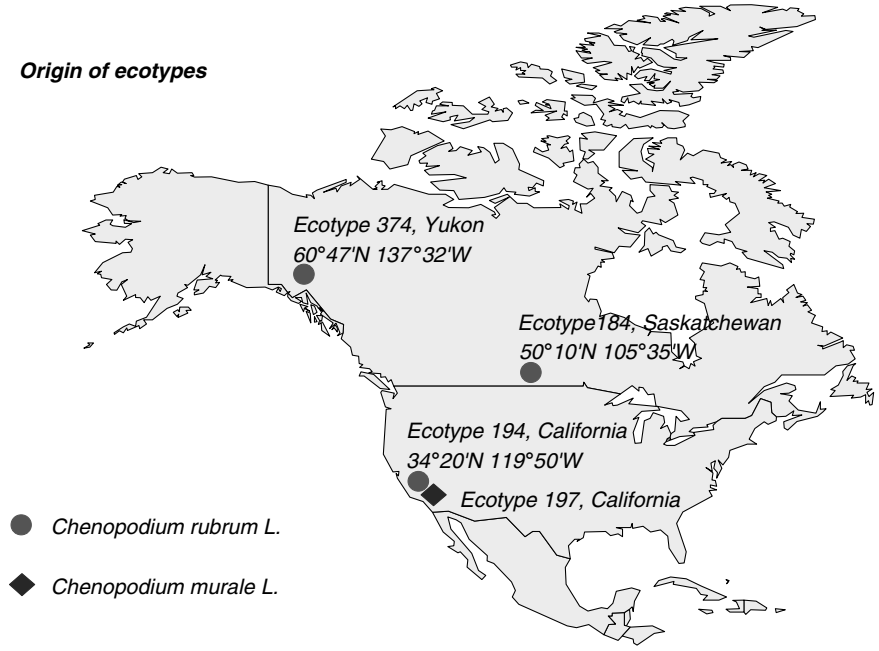

Fig. 10.1 Geographic location of latitudinal ecotypes of *Chenopodium* spp. selected by B.G. Cumming in North America (Cumming 1967). *C. rubrum* L. is a short-day plant, *C. murale* L. a long-day plant

Table 10.1 Period length of circadian rhythms in stem extension rate (growth rate) of *Chenopodium rubrum* L. ecotypes originating from three different latitudes in North America (Cumming 1967), and their specific critical photoperiods for flower induction

Ecotype	194	184	374
Geographical origin	34°20′N	50°10′N	60°47′N
Critical photoperiod	12	16	18
Period length	22.41±0.33	24.34±0.54	27.77±0.71

complex research in circadian and rhythmic processes for many decades. Until recently, the molecular genetic analysis of circadian rhythms in pro- and eukaryotes focussed on transcriptional-translational control loops (TTCL) as a basis for mechanisms of circadian rhythms (CRs). Some doubts, however, have been emerging (e.g. Dunlap 1999; Lillo et al. 2001; Shabala 2006), reviving the view on metabolic control of timing (Hendricks 1963; Wagner and Cumming 1970; Wagner et al. 1975; Albrechtová et al. 2006).

Besides circadian oscillations, living systems display higher- and lower-frequency phenomena in metabolism and behaviour, in signal perception and in communication. In a recent monograph on communication in plants (Baluska et al. 2006), our current knowledge of the total frequency range of biological

rhythms has been summarised in the chapter by Shabala (2006), with special emphasis on ultradian oscillations, i.e. shorter than 24 h. This compact and comprehensive overview highlights the need for future research into functional relationships between ultradian rhythms and the physiology driving plant behaviour.

Metabolic ultradian cycling in yeast seems particularly relevant because it drives a temporal, genome-wide transcription and coordination of essential metabolic processes (Tu et al. 2005). Biochemical and biophysical oscillations seem to be the "hardware" of biological cellular control networks in adaptation to environmental constraints such as the seasonal change in day length. The evolution of the circadian time-measuring principle – this being the basis for the "anticipation" of seasonal change in environmental conditions – shows features of "learning" by adaptation, thereby creating a circadian rhythm "software" (Lüttge 2003). The metabolic control of timing by gating development and behaviour enables sophisticated adaptation of individual cells in cyanobacteria, whole plants or populations of plants (see also Chap. 8 by Hubbard et al., this book). In their competition for survival, a combinatorial interaction (Genoud and Métraux 1999) of input and output variables is becoming ever more evident (Albrechtová et al. 2006), as demonstrated by photoperiodic control of growth and differentiation.

The demonstration of a diurnal rhythm in compound surface-membrane potential (Wagner et al. 1998), and evidence of correlation between action potentials and rhythmic leaf movements as well as stem growth of *Chenopodium rubrum* (Wagner et al. 2006) have prompted us to conceive the circadian oscillation as a hydro-electrochemical phenomenon at the cellular and organismic level. This view is supported by diurnal and circadian regulation of plasma membrane aquaporins (Moshelion et al. 2002). The hydro-electrochemical integration depends strongly on the compartmentation and formation of temporal patterning of calcium ions as key players in signal transduction (Love et al. 2004 see also Chap. 6 by Blatt et al., this book). The basis for temperature-compensated time keeping in the metabolic control nets of transcription and translation, protein synthesis and turnover depends on a circadian rhythm in energy metabolism expressed by ratios in redox and phosphorylation potential. These ratios, termed macroparameters, exert circadian rhythmic control (gating) on two-component signal transduction systems, which can include inputs to and/or outputs from the TTCL (Albrechtová et al. 2006). Redox and phosphorylation potential react and adapt to changing environmental constraints, securing a homeo-dynamic, but flexible, rhythmicity of metabolic processes and gene expression.

10.2 Rhythmicity in *Chenopodium* spp.

Chenopodium sp. was developed as a "petri-dish plant" by Cumming (1959) for large-scale screening of photoperiodic flower induction, several latitudinal ecotypes showing short-day, long-day and day-neutral responses (Cumming 1967; Tsuschiya and Ishiguri 1981). The precise regulation in

photoperiodism displayed by *Chenopodium*, a plant showing obligate photoperiodic control for its short- and long-day ecotypes, and the availability of such ecotypes for further experimentation have led to extensive studies of rhythmic behaviour, flower initiation and energy metabolism (Fig. 10.2;

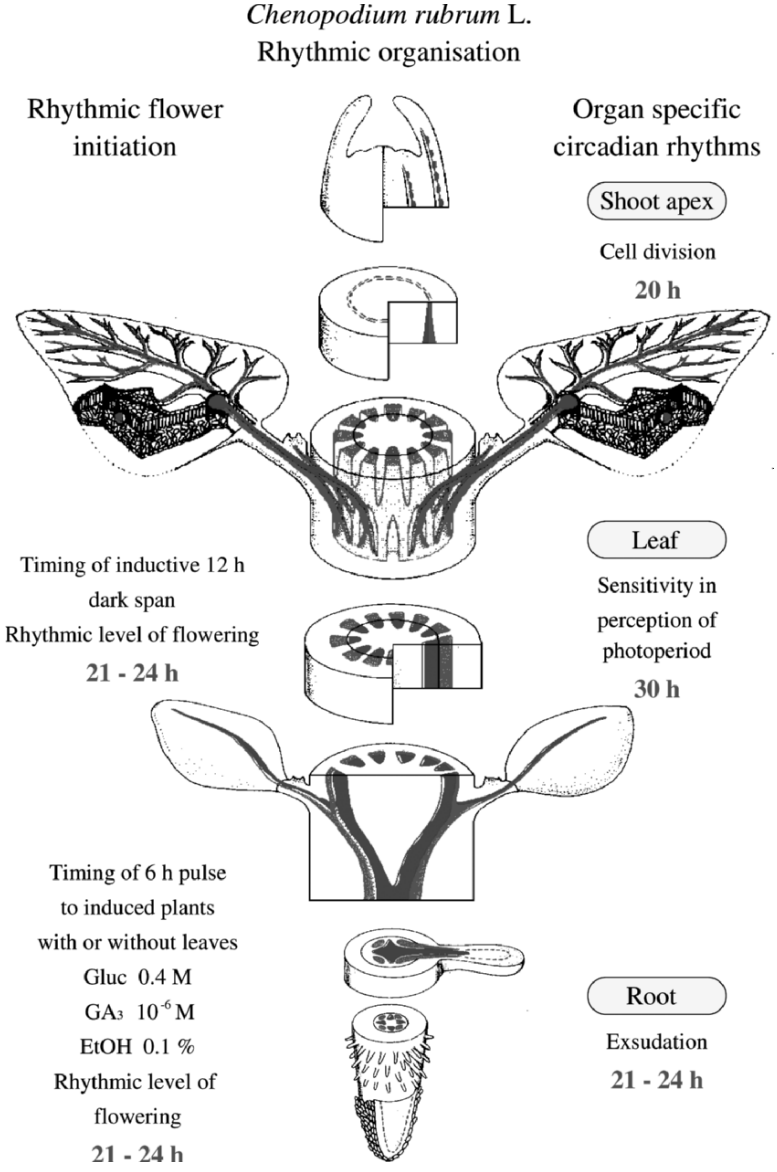

Fig. 10.2 Period length of organ-specific circadian rhythms and photoperiodic control of flowering in *Chenopodium rubrum* L. (after King 1975). Circadian rhythm of root exudation from an isolated root stock of *C. rubrum* (from Wagner et al. 1996)

Wagner et al. 1975; Bünning 1977). The experimental setup used for the quantitative monitoring of stem extension rate (SER) and leaf movement (LM) – as "hands of the clock" – is shown in Fig. 10.3.

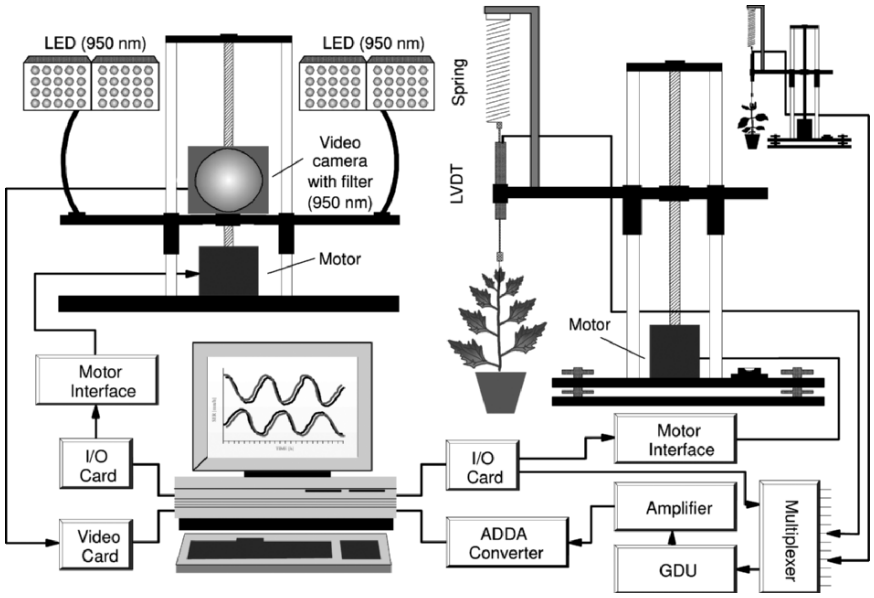

Fig. 10.3 Flow diagram of the experimental setup. The stem elongation measuring and analysing system is based on linear variable differential transformers (*LVDT*). Twelve individual plants and environmental variables such as light intensity and temperature (device not shown) can be measured simultaneously. The leaf movement measuring and analysing system is based on video digitising. Small pieces of aluminium foil are attached as light-reflecting markers to the tip of the leaves, which are viewed from the side. The positions of the markers, visible in the digitised picture as white pixels on a black background, are recorded continuously. To achieve recordings in light as well as in darkness, the markers are irradiated with LEDs of 950 nm; the camera has a filter passing 950 nm light only. Abbreviations: *GDU* generator demodulator unit, *I/O* input/output, *D/A* digital/analog, *A/D* analog/digital. Plant material and growth conditions: seeds of *Chenopodium rubrum* (ecotype 184) and *Chenopodium murale* (ecotype 197) were germinated for 4 days under temperature cycles of 12 h at 32 °C and 12 h at 10 °C either in light–dark cycles of 12 h:12 h (*C. rubrum*) or in darkness (*C. murale*). After germination, the temperature was kept constant at 24 °C and the plants were grown under vegetative conditions, i.e. either they were transferred to continuous light for 21 days (*C. rubrum*) or were kept in light–dark cycles of 5 h light and 19 h darkness for 56 days (*C. murale*). Complete induction of flowering was achieved either by four light–dark cycles of 12 h:12 h (*C. rubrum*) or by 5 days in constant light (C. murale). Prior to the beginning of the experiments, all plants were synchronized with appropriate light–dark cycles. Period lengths were determined by discrete Fourier transformation analysis

10.2.1 Rhythmic Changes in Interorgan Communication of Growth Responses

To develop a coherent view on photoperiodic control in long-day *Chenopodium murale* and short-day *C. rubrum*, the kinetics of SER and LM were investigated as markers of photoperiodic control. Further studies focussed on interorgan communication between the signal-perceiving leaves and the target tissue (stem/apex) implied in the control of flowering.

To study whole-plant behaviour, time-lapse photography was used to investigate rhythmic integration of the main shoot axis and side branches in rhythmic growth as well as in LMs. The SER was continuously monitored by means of an auxanometric system (Ruiz-Fernandez and Wagner 1989, 1994), simultaneously analysing LM via a video system (Fig. 10.3).

An undampened circadian rhythm in the SER was observed in continuous light. Total stem elongation depends on precise cooperation in the stem elongation of single internodes. In one specific experiment dealing with three internodes, the first internode completed growth while the second and third internodes both continued to contribute to total SER. The fraction of stem elongation resulting from growth of the second internode declined while the growth rate of the third internode increased. These two internodes thus displayed individual rhythms in SER with the same phasing and a reciprocal change in amplitude, resulting in a stable additive amplitude and showing an undamped oscillation (Lecharny and Wagner 1984; Lecharny et al. 1985). In the elongating stem, the internodes sustain an undamped circadian rhythm in SER as they move upwards.

Both short- and long-day plants exhibit a precise circadian rhythmicity in SER, in vegetative plants with period lengths of 24.34±0.54 h (*C. rubrum*, n=24) and 27.47±0.57 h (*C. murale*, n=28) respectively. Flowering plants show a significantly shorter period length in SER, the corresponding values being 23.44±0.76 h (*C. rubrum*, n=24) and 26.56±0.68 h (*C. murale*, n=56; Table 10.2) respectively. The period lengths of LM mirror the kinetics in SER, displaying similar increases of frequency in the flower-induced state. Whereas in vegetative plants of *C. murale* the kinetics of SER and LM are 180° out of phase, this phase relationship is shifted after flower induction (Fig. 10.4). Both parameters display clear movement and growth patterns with photoperiod-specific reactions to "light-on" and "light-off" signals. The pattern of overall growth kinetics varies considerably depending on the photoperiod (Figs. 10.5 and 10.6). To analyse this patterning quantitatively, the ratios of integral growth in dark over integral growth in light were calculated. A distinct relation with the induction of flowering was observed, from which a threshold specific for each short-day (SDP) and long-day plant (LDP) could be determined. Flower induction results in a threshold value of stem growth of 0.6 for *C. rubrum* and 4.0 for *C. murale* for the ratio [integral growth during the dark period]:[integral growth during the light period], as shown in Fig. 10.7a, b.

The above observations also hold true for the kinetics of leaf movements. This is hardly surprising, as rhythmic LMs in *Chenopodium* spp. do not

Table 10.2 Organ-specific period lengths of leaf movements and stem extension rates of flowering and vegetative plants of *Chenopodium rubrum* and *Chenopodium murale*, either intact or continuously defoliated (*n* denotes sample size)

	Vegetative	Flowering
Stem extension rate		
Chenopodium rubrum L.		
Intact	24.34±0.54 h (*n*=24)	23.44±0.76 h (*n*=24)
Defoliated	22.46±0.57 h (*n*=10)	22.80±0.54 h (*n*=4)
Chenopodium murale L.		
Intact	27.47±0.57 h (*n*=28)	26.57±0.68 h (*n*=56)
Defoliated	23.85±0.39 h (*n*=12)	23.81±0.69 h (*n*=4)
Leaf movement		
Chenopodium rubrum L.		
Intact	23.68±0.60 h (*n*=5)	22.78±0.72 h (*n*=5)
Chenopodium murale L.		
Intact	27.03±0.64 h (*n*=16)	26.60±0.98 h (*n*=27)

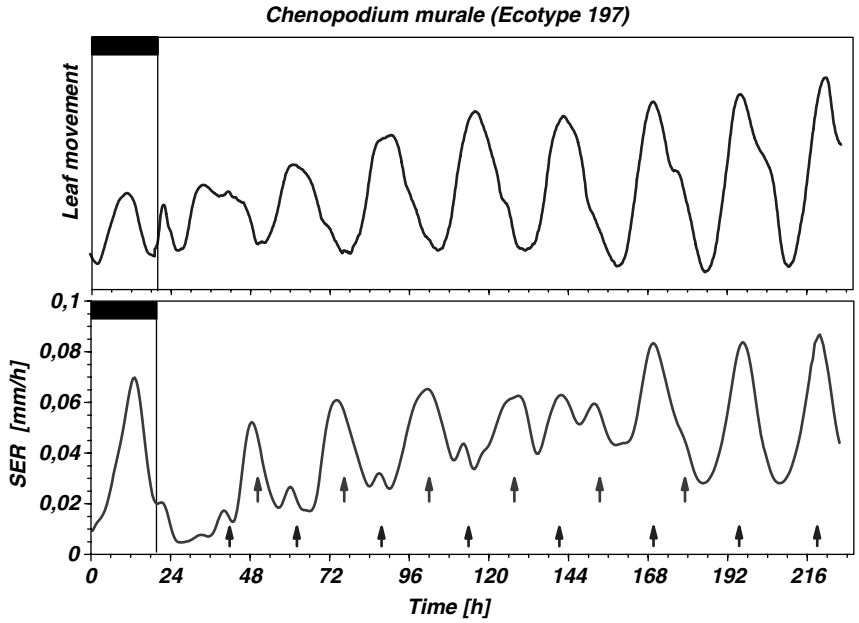

Fig. 10.4 Comparison of leaf movement (*upper graph*) and stem extension rate (*SER*, *lower graph*) of vegetative *Chenopodium murale* being induced to flower during exposure to constant light. The *lower arrow line* in the lower graph indicates a component of the oscillatory pattern which is out of phase with the oscillation in leaf movement and detectable only in vegetative development. The *upper arrow line* indicates the component of the oscillatory pattern which is in phase with leaf movements and becomes the dominant oscillation after the plants have been irreversibly induced to flower (between 144 and 156 h)

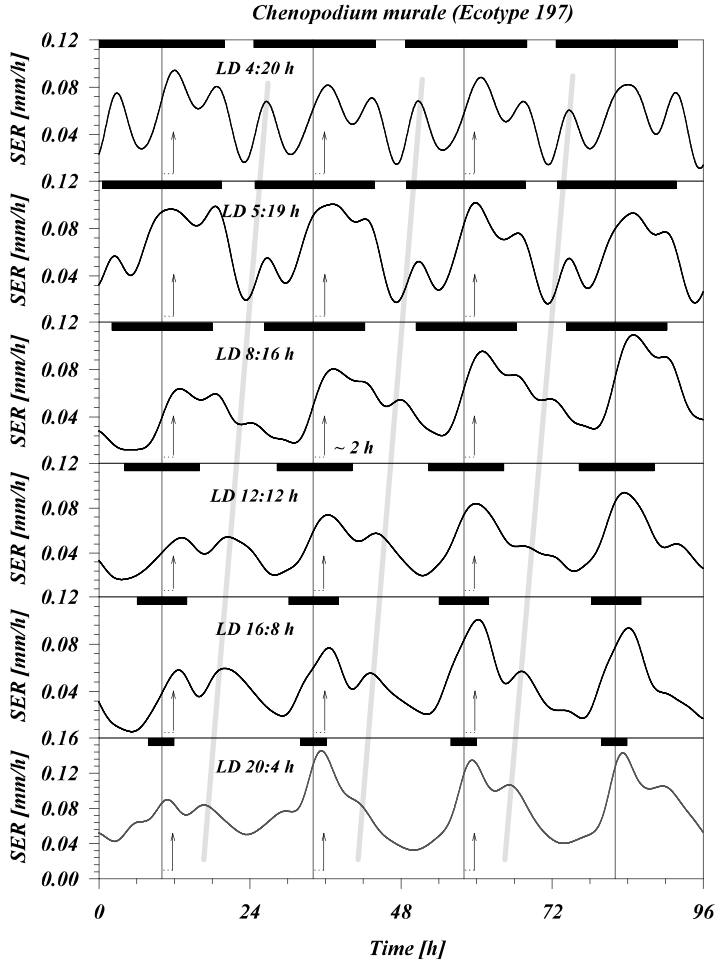

Fig. 10.5 Kinetics of stem extension rate (*SER*) of *Chenopodium murale* in different photoperiods. The kinetics are scaled to the middle of the dark period (*black horizontal bars*). The *arrows* indicate peaks appearing ~2 h after the middle of the dark period. *Grey vertical bars* indicate the position of the peak appearing 6–8 h after the onset of the light period (cf. Fig. 10.6)

depend on differential turgor changes of flexor and tensor cells (Satter and Galston 1973 see also Chap. 1 by Moran, this book). There are no pulvini at the base of the petioles. The LMs are due to the timing of differential growth at the upper and lower surface of petioles and leaf base. A detailed observation of time-lapse movies clearly shows that the rhythmic folding up of leaves starts at the uppermost leaves surrounding the apical bud, progressing down the stem axis to the lowest still growing pair of leaves. The rhythmic LMs reflect rhythmic changes in hydraulics. Such changes in hydraulics are also evident at the basal end of the plant, in the form of a circadian rhythm in root exudation (Fig. 10.2). A hydraulic–electrochemical integration seems to be the basis for interorgan communication in plants.

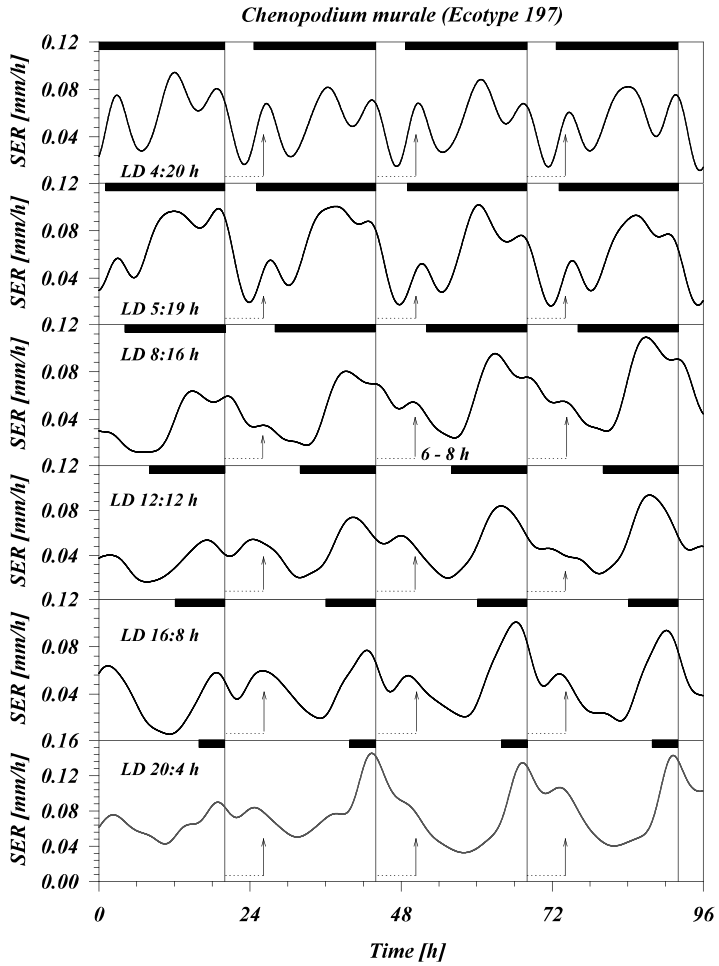

Fig. 10.6 Kinetics of stem extension rate (*SER*) of *Chenopodium murale* in different photoperiods. In LD 4:20 h and LD 5:19 h, the plants remain vegetative whereas they are induced to flower in LD 8:16 h, LD 12:12 h, LD 16:8 h and LD 20:4 h. The kinetics are scaled to the onset of the light period (*black horizontal bars* represent dark periods). The *arrows* indicate peaks appearing 6–8 h after the onset of the light period

10.2.2 Local Hydraulic Signalling: the Shoot Apex in Transition

Photoperiodic flower induction involves a reorganisation of organogenesis at the shoot apical meristem (SAM) – from vegetative phylotaxis to floral development. Floral transition includes molecular signalling as well as physiological and physical changes. Each step in molecular signal transduction is influenced by feedback from sequences of events from other pathways (cf. Genoud and Métraux 1999 see also Chap. 8 by Hubbard et al., this book).

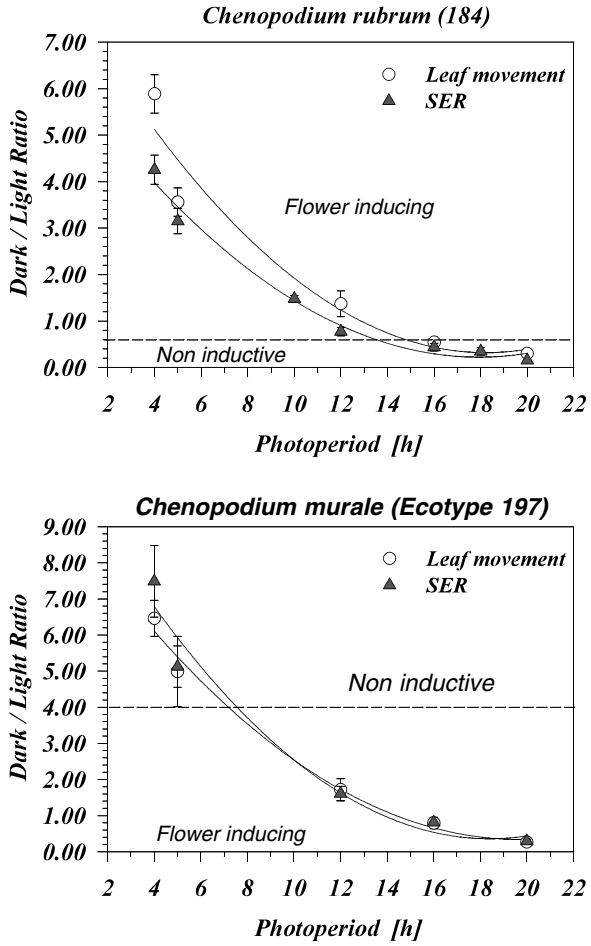

Fig. 10.7 Kinetics of the ratio of integral growth during the dark period over integral growth in the light period for the stem extension rate (*SER*, *triangles*), and the corresponding ratio for leaf movement (*circles*) under various photoperiods. In *C. rubrum* (**a**), flower-inducing photoperiods are correlated to ratios above 0.6 (*dotted line*) whereas, in *C. murale* (**b**), flower-inducing photoperiods are correlated to ratios below a threshold value of 4 (*dotted line*)

In this way, the "physiological state" of a plant controls signal transduction, and vice versa. Very little is known regarding relationships between the molecular and physiological levels of the control of organogenesis, such as stem elongation or leaf and flower production at the SAM. To analyse the change from the vegetative to the flowering apex, we studied the kinetics of molecular and physiological changes at the SAM of *C. rubrum* during floral transition, aiming at elucidating the kinetic relationships between these events.

Physical strain at the surface of the SAM was previously suggested to play a key role in the patterning of organogenesis (Green 1994). The distribution of local forces is a result of the upward pressure from the expanding inner cell layers (corpus) to the sheet on the surface (tunica), and from the local extensibility of cell walls at the surface. A signalling network regulates organogenesis, involving molecular, biophysical and biochemical pathways of signal transduction (see also Chap. 2 by Moreno et al., this book). During phase change from vegetative to reproductive development, the communication between the leaf, stem and SAM changes (see Table 10.2, Fig. 10.2). These changes, as seen for the ratios in SER and LM described above, are reflected in the kinetics of SER in response to different photoperiods inductive or not for flowering (Fig. 10.7a, b). There is a specific timing of peaks and troughs in SER establishing specific phase relationships with respect to photophile and scotophile time spans in diurnal photoperiodic cycles, showing an essentially perfect temporal integration of cell division, growth and differentiation.

Our studies are intended to increase understanding of local water transport and turgor changes as related to changing organogenesis at the SAM of *Chenopodium* plants under photoperiodic flower induction. We showed that the size of the SAM increases during flower-inducing treatment (Albrechtová and Wagner 2004; Albrechtová et al. 2004). The expansion of the meristem results from cell enlargement, rather than changes in cell division, and therefore is presumably based on water uptake. The phylotactic spiral changes to a circular pattern, the latter visible as local differences in the optical properties of cell walls. The results suggest that organogenesis changes long before flower induction is completed. We therefore conclude that the change of organogenesis at the SAM during floral transition is initiated by an increased movement of water into the SAM, leading to its expansion and to the redistribution of forces at its surface.

Seeing that a change in water movement can involve aquaporins, these were subsequently studied in the SAM of *C. rubrum*. Aquaporins (AQPs) are highly selective water channels facilitating transport of water across the membrane (Baiges et al. 2002). In *C. rubrum*, a new gene, *CrAQP*, has been identified which shows a high homology to aquaporins found in other plants (Albrechtová and Wagner 2004). In leaves and the SAM, its expression differs significantly between vegetative and flowering plants (Albrechtová and Wagner 2004). Involvement of aquaporins at the SAM in flower initiation was proven by applying an inhibitor of aquaporin activity, HgCl, directly to the SAM. When applied before or during the dark period, HgCl partially inhibited flowering.

A comparison of the kinetics of parameters studied revealed that the increase in SAM size is accompanied by an increase in calcium concentration and average pH value at the SAM (Walczysko et al. 2000; Albrechtová et al. 2001, 2003). Further work will reveal whether intracellular pH and calcium concentration can influence water transport by regulating activity of *CrAQP* (cf. Tournaire-Roux et al. 2003; Love et al. 2004). Other observations have also confirmed a putative increase of free sucrose at the SAM during floral transition

(Albrechtová and Wagner 2004). The increase in sucrose concentration could lead to an increase in osmotic pressure in SAM cells, thereby producing – together with a redistribution of ions – a driving force for water transport.

A central role in the shift of organogenesis during floral transition is thus played by the water status, the local physical properties of cell walls and the distribution of local forces at the surface of the meristem. In combination, these results support the hypothesis of the involvement of hydraulic signals in organogenesis at the shoot apical meristem. It is anticipated that hydraulic changes at the SAM leading to flower initiation are mediated by specific hydro-electrochemical communication between the leaves and SAM (and roots). All organ systems show rhythmic activities with specific phase relationships (cf. Fig. 10.2).

Turgor-dependent volume changes, stretch-activated membrane channels (Kung 2005) and correlated changes in membrane potential might all be an essential part of the "hardware" for signal transduction at the cellular and organismic level. The "software" could involve frequency-coded signals at the cellular, tissue and organism level (see also Chap. 6 by Blatt et al., this book).

10.2.3 Membrane Potential as the Basis for Hydro-Electrochemical Signalling, Interorgan Communication and Metabolic Control

Rhythmic integration of processes over the whole plant possibly involves modulation of turgor pressure via stretch-activated ion channels (Pickard 1973) and aquaporins, with concomitant changes in membrane potential. Accordingly, maintaining the integrity of the plasma membrane might form the basis of hydro-electrochemical activity reflected in action potentials, as discussed by Goldsworthy (1983).

Membrane potentials (typically, −100 mV, cytosol negative) are maintained by the activity of electrogenic ion pumps and provide the energy for the active transport of many substances across the membrane. Depolarization of cells at the level of the plasma membrane may evoke action potentials propagating along the organism. Goldsworthy proposed that action potentials might have evolved as a mechanism for rapidly switching off the membrane potential of cells to enable repair of a damaged cell membrane without excessive loss of ions from a localised injury. The generation of action potentials in plant cells would depend on the sensitivity of cells, so that permeability of ions is increased by turgor-mediated mechanical deformation. Such changes depend on the metabolic activity of the cells, leading to a change in turgor with subsequent modifications in the activity of mechano-transductive ion channels (Lang and Waldegger 1997; Kloda and Martinac 2002).

Changes in surface-membrane potential were investigated using bipolar recordings with surface platinum electrodes. On the basis of the early changes observed at the apex and of our measurements of changes in electrical activity during flower induction, we suggested that the signals for flowering might

be transmitted from the leaves to the apical meristem via hydraulic–electrochemical impulses (Albrechtová et al. 2005). A similar mode of action has been shown in systemic wound reactions (Wildon et al. 1992).

A diurnal rhythm in the resting surface potential of the plasma membrane (Wagner et al. 1998) possibly reflects the daily change in photophile and scotophile phases (Bünning 1977). This rhythm has its origin in a circadian rhythm in energy metabolism, and is probably the basis for circadian-rhythmic changes in sensitivity to signal perception, signal generation and signal transduction. It has been proposed that the communication between plant organs (leaves, shoot apex, roots) involves frequency-coded (electric) signals (Wagner et al. 1998). Furthermore, spontaneous compound surface-membrane action potentials were shown to correlate with turgor-controlled hydraulic growth movements of leaves and stem extension rate, which are controlled by the photoperiod. Due to its mechanisms of action, hydraulic and electric signalling is intrinsically coupled (Wagner et al. 2006) see also Chap. 2 by Moreno et al. and Chap. 5 by Johnsson, this book.

10.3 Conclusions and Perspectives: Rhythms in Energy Metabolism as Determinants for Rhythmic Growth and Leaf Movements

Rhythmic changes in energy metabolism, as displayed in *C. rubrum*, might be the essence of circadian oscillators not only in higher plants but also in cyanobacteria (Wagner et al. 1975; Albrechtová et al. 2006). Circadian rhythms in redox and phosphorylation state are considered to be gating parameters originating from energy metabolism in an adaptive strategy of living systems to changes in daily light–dark cycles. These considerations support the view that action potentials and the circadian rhythmic organisation of energy metabolism are very early achievements to avoid oxidative damage during light conditions (Fig. 10.8).

From a detailed analysis of rhythms in enzyme activities involved in compartmental energy metabolism with and without feeding of sugars (cf. Frosch and Wagner 1973; Frosch et al. 1973; Wagner et al. 1975; Jang and Sheen 1994), and of changes in nucleotide pool size levels in the short-day plant *C. rubrum*, we compiled evidence supporting circadian rhythmicity in overall energy transduction. Circadian rhythmic energy metabolism would be the result of a compensatory control oscillation between glycolysis and oxidative phosphorylation, coupled to photophosphorylation in cyanobacteria (Huang et al. 1990) and green plants (Wagner and Cumming 1970; Wagner et al. 1974a, b; Wagner 1976a–c). Thus, circadian rhythmicity could involve energy control of ion transport processes at the membranes of cells and organelles. The membrane's physical state, e.g. modulated by temperature, could control transcription (Vigh et al. 1998) or, via frequency-coded calcium oscillations,

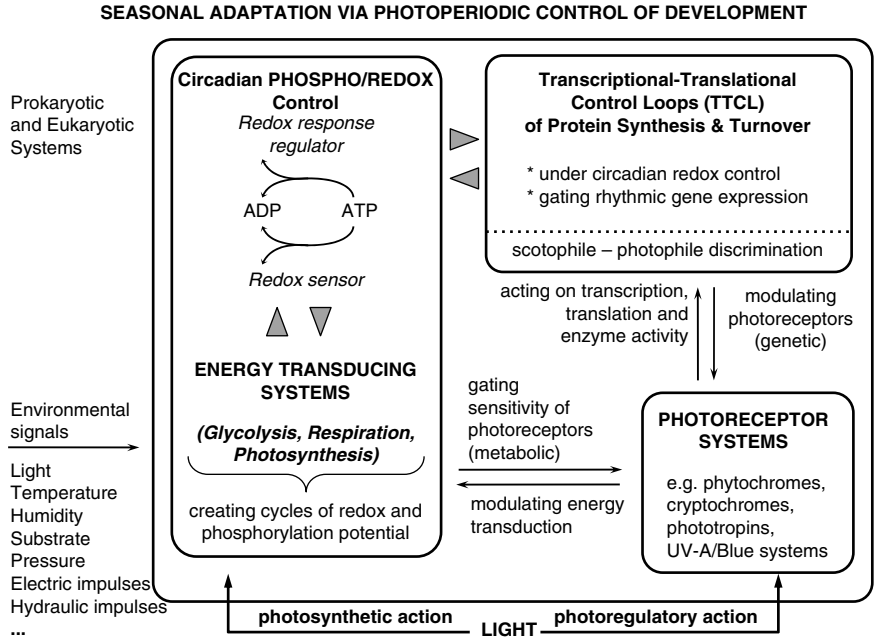

Fig. 10.8 Flow diagram of the postulated regulatory network involved in the generation of circadian regulation of metabolism and behaviour at the cellular level. The regulatory loops generate a circadian rhythm in energy transduction (Wagner and Cumming 1970; Wagner et al. 1975), resulting in cycles of redox and phosphorylation potential, and a rhythmic membrane potential (Wagner et al. 1998) which controls circadian rhythmic changes in sensitivity of membrane-bound photoreceptors. In addition, and more generally, membrane-bound (e.g. two-component) signal transduction systems (Hwang et al. 2002) not only synchronize the circadian rhythm in energy transduction with the daily light–dark cycle via several photoreceptor systems but also modulate metabolic control of timing by many other environmental signals or parameters of state. Redox and phosphorylation potentials gate the input of transcription factors to an autoregulatory cycling of transcription and translation, protein synthesis and turnover. This forms the basis for the evolution of latitudinal ecotypes of *Chenopodium rubrum* exhibiting specific period lengths for their circadian rhythms (see Table 10.1), and the separation of the circadian cycle into specific photophile and scotophile elements for photoperiodic control. By scanning the seasonal changes in the daily light–dark cycle for coincidence with the ecotype-specific scotophile–photophile pattern, the critical photoperiod switches the system from vegetative to reproductive development

lead to differential gene activation and expression (Dolmetsch et al. 1998; Li et al. 1998, Love et al. 2004 see also Chap. 6 by Blatt et al., this book).

Taking into account the symplastic organisation of higher plants and phloem conductance (Lough and Lucas 2006), the concept of compartmental feedback becomes even more attractive in view of bioelectric phenomena. This symplastic organisation might be the basis for the translocation of electric, photoperiodic and morphogenetic stimuli (Genoud and Métraux 1999), so that proton translocation and concomitant ion movements would give rise

to a circadian rhythm in electric potential, paralleled by circadian rhythms in growth, like leaf movements and stem extension rates as universal markers of systemic behaviour (Aimi and Shibasaki 1975; Wagner et al. 1998).

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11 Rhythms and Morphogenesis

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Abstract

Most plants are constructed from repeating units such as phytomers, merophytes and cell packets. Even simple organisms such as the filamentous blue-green alga *Anabaena* show repeating cellular structures, notably with respect to the distribution of their heterocysts. All these are examples of the workings of inherent rhythms of development. Here, these rhythms are discussed from the point of view of L-systems and Petri nets which, although theoretical devices, can bring insights into the possible biological components or processes which establish the rhythms.

11.1 Introduction

More than 100 years ago, Charles Darwin and his son Francis found themselves fascinated by the oscillations of roots, shoots and tendrils, and these they meticulously described in their book 'The power of movement in plants' (Darwin and Darwin 1880). Their view was that the growing points of plants as well as their newly produced branches were in a state of continuous rotation, or else they manifested some other form of rhythmic agitation. With the assistance of sophisticated methods of recording and analysing organ growth (e.g. Iwamoto et al. 2006), some of the physical and physiological bases of these oscillations are now becoming better known. Although many of the Darwins' descriptions related to oscillations of either full-grown or actively growing plant parts, rhythmic and periodic phenomena are now also known to accompany the genesis of new organs at the plant's meristematic apices. The importance of these organogenetic rhythms is that they lead directly to a repetitious, or modular, type of construction (Notov 2005) which, for higher plants, is vital for their reproduction (sexual and asexual) and their vegetative movements (for example, shoot tropisms involving the repeatedly produced

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nodes which comprise the stems of grasses). However, rhythmic formatory activities leading to the development of modular constructions are also found in 'lower' organisms, such as the cyanobacteria (blue-green algae). In this chapter, we shall describe some examples of rhythmic morphogenesis in both simple cyanobacteria as well as in more complex higher plants.

Fundamental to analyses of the regulation of growth rhythms in plants is a representation of the organism as it exists in time. Therefore, it is necessary to be sure that the interval between observations does not lead to loss of any information which might shed light on the regulatory factors of development (Barlow and Powers 2005). Moreover, as will become evident in the present chapter, we contend that it is desirable to adopt some theoretical framework which provides a context for the observations made, and which may also, in time, provide a general model for certain types of organogenetic phenomena in plants.

11.2 Developmental Theories and Their Application to Rhythmic Morphogenesis

What are these theoretical frameworks and, importantly, what do they contribute to the study of the rhythms of plant development? In the present case, we have made use of two methodologies for the exploration of theoretical pathways of development, which can then be juxtaposed with actual observations: L-systems (Lindenmayer 1971; Lindenmayer and Jürgensen 1992) and Petri nets (after Petri 1980; Brams 1983).

L-systems handle the transformation of states which, in the present context, are held to be comprised of a set of biological features which may be morphological, physiological or structural. The transition between one state and another occurs during a timestep. When coupled with growth, state transformations comprise the basis of organismic development. If the transitions are deterministic – that is, if one state is invariably followed by some other particular state – and if a series of states and their transitions are recurrent, then a cyclical or oscillating system becomes manifest. The sequence of developmental changes which emerge is cumulative: the results of former state transformations are not erased but are added to at each timestep, thereby bringing about a gradual 'metamorphosis' of organic form. The L-systems discussed in the present chapter are deterministic (D) and interactionless (0) or with interaction (I), i.e. D0L-systems or DIL-systems, respectively. The timescale of development is defined by the timesteps which, in turn, are related to the discreteness of the steps chosen as representative markers of change.

A Petri net is a set of potential states (represented, in this context, by places or conditions, but otherwise a 'state' is defined as above for L-systems), events and connecting arcs. A simple net, as opposed to a stochastic net, has

a determinism similar to that of an L-system. In more detail: a given state of an entity (or place), be it that of a molecule, a cell or a whole plant, is held to contain some property which determines it as a precondition for the achievement, by means of an event, of a new state of that entity (J. Lück and Lück 1991). Hence, a state which earlier was the precondition for an event becomes, when all other contingent conditions are fulfilled, the postcondition of that same event; and this postcondition is now set to become a precondition for a future transitional event, and so on. Thus, within a net, numerous possible states exist, and these may (or may not) trigger any one of a number of events which then leads to the development of a new set of states. The gradual unfolding of the net, via the actualising of a succession of potential states, corresponds to the development of a biological system at a particular level of organisation. This can lead to the disclosure of vast networks of gene interactions (e.g. Menges et al. 2002) and metabolomic compartments (e.g. Morgenthal et al. 2006) as well as metastable morphological states (J. Lück and Lück 1991), which together drive forward the life cycle. A Petri net is considered to be 'closed' if a particular set of states reappears after a sequence of events. Recurrence of states, as invoked by the net, would be a representation of rhythmic behaviour within the analogous biological system. For the orientation of the reader, a very simple Petri net is shown in Fig. 11.6, though discussion of this net is deferred until later in the chapter.

A Petri net does not trace the flow of information; its vector records only the stages of development passed through by the system. Although growth may be implied, the arcs of the net indicate only which events are possible. (In this way, they contrast with L-systems where all symbols present at a timestep have to change their state in parallel.) The successive filling and emptying of places (conditions) within the net show the sequence of states which arise from an initial event, rather as ripples move over the surface of water following a perturbation.

11.3 Rhythmic Patterns of Cellular Development Within Cell Files

The cell is generally thought of as an immensely complex unit which is fundamental to the phenomenon known as 'life' (Mitchell 1911). Aggregations of cells which arise as a result of cellular self-reproduction and division are commonly held to construct living organisms. The individual cells which construct the multicellular states contained within the body of the organism are arranged in space and time in accordance with an inherent organismal Bauplan. The multicellular state itself, with its various groups of tissues and organs, is then enabled to organise an efficient 'division of labour' for the task of living – that is, for perpetually transforming itself and its environment, as well as for its autoreproduction.

We shall now explore a simple example, taken from a filamentous blue-green alga, to illustrate the rhythmic patterning of cell division and differentiation which maintains its form. The processes can be revealed with the aid of L-systems. Despite their apparent simplicity and perceived primitiveness, the cyanobacteria reveal many of the organismal traits found in higher plants: structured meristems, distinctive apical cells, ordered branching patterns, differential growth, and so on (Geitler 1960).

The cyanobacterium, *Anabaena catenula*, provides an example of a simple developmental rhythm. This organism has a filamentous form; it is comprised of a uniseriate multicellular trichome enclosed within a mucilaginous sheath. Divisions of the vegetative cells (v) which constitute the trichome are unequal, one daughter, v_1, being approximately 20% shorter than the other daughter, v_2, thus giving a cellular birth-length ratio of 5:4 (Mitchison and Wilcox 1972). At the level of the organism, there is an axiality which maintains the filamentous form; in turn, this results from (or ensures) the anisotropic growth of the cells. The realization that v_1 and v_2 cells have a particular distribution along *Anabaena* filaments led Mitchison and Wilcox (1972) to propose a rule governing the division of the v cells. Later, the cell division system of *A. catenula* was described in a formal way by Lindenmayer (1982) using a D0L-system algorithm. However, the algorithm used in that work pointed to a 2:1 ratio between the life spans of v_1 and v_2. Because a 5:4 birth-length ratio gives a closer correspondence to the actual cellular life spans, we take as more precise the following L-system algorithm, *A*1:

$$
\begin{array}{lcll}
\vec{v}_1 & \rightarrow & \vec{v}_2 & \\
\vec{v}_2 & \rightarrow & \vec{v}_3 & \\
\vec{v}_3 & \rightarrow & \vec{v}_4 & \\
\vec{v}_4 & \rightarrow & \vec{v}_5 & \\
\overleftarrow{v}_1 & \rightarrow & \overleftarrow{v}_2 & A1 \\
\overleftarrow{v}_2 & \rightarrow & \overleftarrow{v}_3 & \\
\overleftarrow{v}_3 & \rightarrow & \overleftarrow{v}_4 & \\
\overleftarrow{v}_4 & \rightarrow & \overleftarrow{v}_5 & \\
\vec{v}_5 & \rightarrow & \overleftarrow{v}_2\,\vec{v}_1 & \\
\overleftarrow{v}_5 & \rightarrow & \overleftarrow{v}_1\,\vec{v}_2 &
\end{array}
$$

As mentioned above, short and long daughter cells of a division are denoted as v_1 and v_2. The overlying small arrows $\leftarrow$ and $\rightarrow$ refer to the polarity of the indicated cell. This polarity is the outcome of a cell division event which is achieved when the cell attains state v_5. The combination of cell type (either v_1 or v_2) and polarity defines the state of each cell. States transform as directed by the large arrows ($\rightarrow$). The outcome is the rhythmic, and recurrent, production of a certain pattern of v cells and their states along the filament (Fig. 11.1).

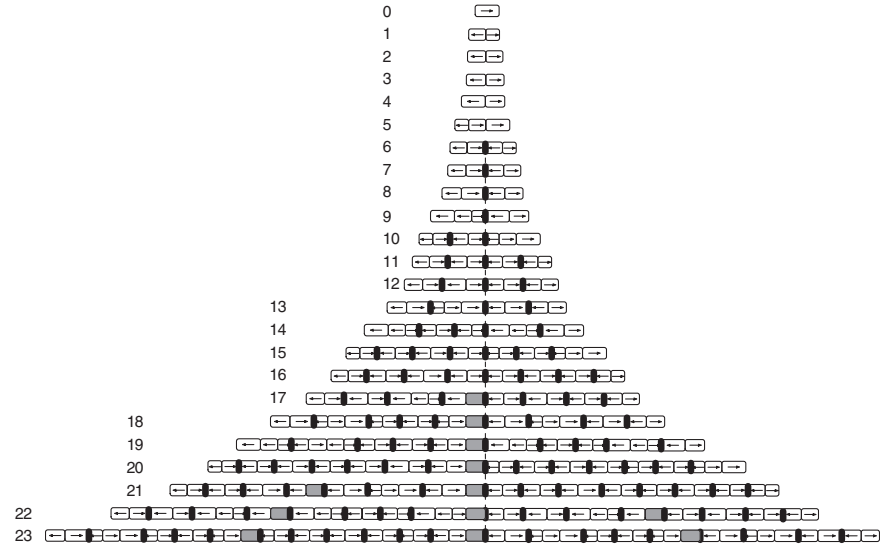

Fig. 11.1 Development of a uniseriate filament of *Anabaena catenula*. Commencing with vegetative cell $\vec{v}_5$, a series of cells develops over i=23 timesteps (up to i = 16 in accordance with algorithm $A1$). A 5:4 ratio between life spans of v_1 and v_2 cells was adopted. Polarities of the v cells are indicated by *arrows*. Heterocysts are the *shaded cells* and arise from v_1 cells. Here, they are spaced in accordance with the transition rules of algorithms $A1$ and $A2$; the spacing is also in approximate conformity with published data. Thickened partitions between cells are septa which confront opposing cell polarities. To the *left* of these, a new heterocyst will eventually form

Commencement of growth from a $\vec{v}_5$ cell leads to a particular orientation of trichome development. At the stage when a trichome has 46 v cells (at timestep i=23 in Fig. 11.1), 25 of these are to the left side of the midline defined by the partition between the first pair of daughter v cells, and 21 v cells are to the right side of the midline. This arrangement of cells could be the basis of another polarity – one inherent to the filament. In this particular case (see Fig. 11.1), let us say that filament polarity is from left to right. This filament polarity is the consequence of the polarity of the $\vec{v}_5$ cell which initiates the descent. If the asymmetry of division were more marked – with, for example, a 2:1 ratio of timesteps leading to $\vec{v}_2$ and $\vec{v}_2$ cell divisions, respectively – this would result in 21 and 13 cells on either side of the midline after only i=7 timesteps.

Linked with this system of unequal, or asymmetric, cell divisions is a rhythmic pattern of cell differentiation characteristic of certain taxa of blue-green algae. In the case of members of the Nostocales (of which *Anabaena* is a member) and the Stigonematales, one major differentiation event involves the transformation of a dividing, vegetative v cell into a non-dividing heterocyst H. This process is preceded by a 'determination' event within a particular v cell

whereby its differentiation as an *H* cell becomes inevitable. A simple proposal is that, after a certain number of timesteps, a descendent of a v_1 cell is determined as a proheterocyst, *h* (Wilcox 1970). Differentiation of an *H* cell is completed after a few further timesteps. This same pattern of development is repeated throughout the trichome. A question dealt with below is which of the numerous v_1 cells is selected for proheterocyst determination. (Note, however, that v_1 cells at the ends of a filament do not differentiate as *H* cells.) The same question can also apply to the differentiation of idioblasts taking place within certain cell files of higher-plant tissues (Barlow and Lück, unpublished data).

Observations on the rhythmic formation and, hence, regular spacing of *H* cells (Wilcox 1970; Mitchison and Wilcox 1972) have given rise to the idea that *H* cell determination is regulated by an inhibitor, the source of which is an existing heterocyst (Mitchison et al. 1976; Golden and Yoon 2003). It is supposed that each new *H* cell derives from a vegetative *v* cell at a position along the filament where inhibitor concentration has fallen below a certain value, whereupon genes for cyst differentiation become operative. Thus, the rhythmic pattern of *H* cell differentiation can be envisaged as being the result of a rhythmic oscillation of morphogenetic gradients along the trichome. This proposal has been modelled by De Koster and Lindenmayer (1987) who were able to combine diffusion equations with L-system cell productions.

We propose an alternative to this model. Moreover, it is a model which uses elements in keeping with other L-system models for cell division and differentiation and, in this respect, it conforms to a general class of L-system-based models – to uncover these is also one of the goals of our theoretical pursuit. Central to the model is a particular spatiotemporal distribution of the division septa inserted between vegetative *v* cells. Septum age is also presumed to bear a close relationship to *H* cell differentiation. In order only to minimise the number of terms needed for a description of developmental events, we take the example of a 2:1 ratio of daughter cell birth-length (or division rates; Fig. 11.2). A $\vec{v}_2$ cell initiates the cellular descent. At the cyanobacterial 'mitosis' and division (Haupt 1923), a septum begins to grow centripetally from the mother cell's side walls. This marks the start of the formation of two daughter cells, v_1 and v_2. The septum requires a certain time, here assumed to correspond to i=2 timesteps, for its growth to be completed. Accordingly, the state transitions of *A*1 have been both simplified to a 2:1 length ratio and extended to accommodate heterocyst formation, as follows in the set *A*2.

$$
\begin{aligned}
\vec{v}_1 &\rightarrow \vec{v}_2 \\
\vec{v}_1 &\rightarrow \vec{v}_2 \\
\vec{v}_2 &\rightarrow \vec{v}_1 \,|_1\, \vec{v}_2 \\
\vec{v}_2 &\rightarrow \vec{v}_2 \,|_1\, \vec{v}_1 \\
|_1 &\rightarrow |_2
\end{aligned}
$$

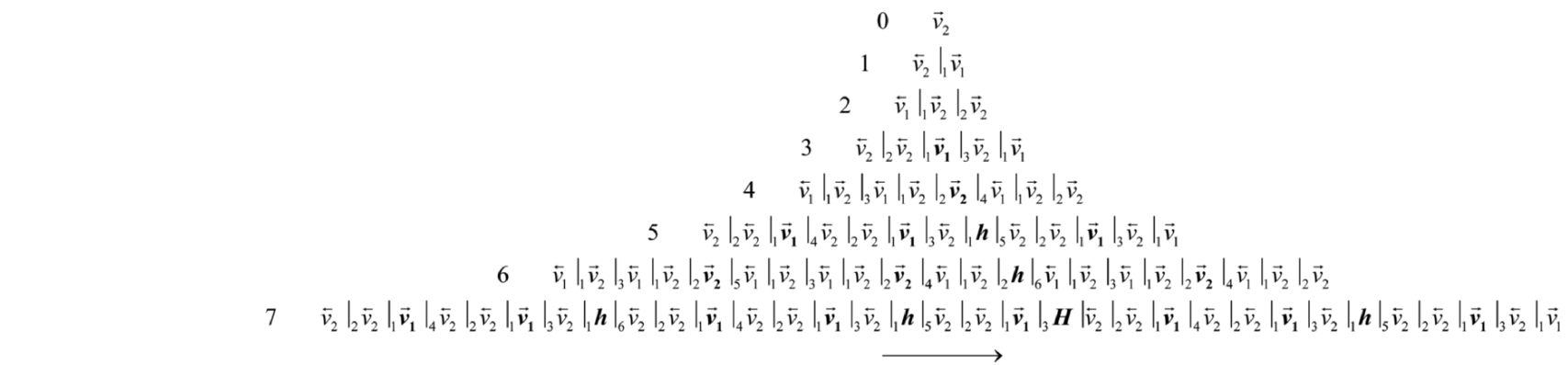

Fig. 11.2 Theoretical development of a cellular filament of *Anabaena* similar to that depicted in Fig. 11.1 but developed over only i=7 timesteps (these steps are indicated by *numerals at the left* of each file) and with a 2:1 ratio of life spans for the v_1 and v_2 cells, respectively. The system commences with cell $\vec{v}_2$ and proceeds according to the rules of algorithm *A*2. Besides the identification of the cells and their polarities, the ages of their septa ($|$) are also indicated (subscript numerals), following their initiation at timestep 1 (e.g. $|_1$). At timestep i=3, a septum ($|_3$) is ready to support determination of a proheterocyst, *h*. At timestep i=5, the septum is mature ($|_5$). Mature septa older than i=6 have no further indices. When the septa at the apical end of a cell is at or beyond these two states, the heterocyst is differentiated (*H*). The cell v_1 is that which gives rise to the proheterocyst, *h*. The *arrow* at the base of the figure indicates the left-to-right (base–apex) polarity of the filament

$$
\begin{aligned}
|_2 &\rightarrow |_3 \cdots \\
|_6 &\rightarrow | \qquad\qquad \text{A2} \\
| &\rightarrow | \\
\vec{v}_2|_2 \ \textbf{\textit{or}} \ \vec{v}_2|_3 &\rightarrow \vec{v}_2|_1\vec{v}_1 \\
\vec{v}_1 &\rightarrow \vec{v}_2 \\
\vec{v}_2 &\rightarrow \vec{v}_2|_1 \boldsymbol{h} \\
\boldsymbol{h}|_5 &\rightarrow \boldsymbol{h} \\
\boldsymbol{h}|_6 &\rightarrow \boldsymbol{H} \\
\boldsymbol{H} &\rightarrow \boldsymbol{H}
\end{aligned}
$$

Each rule, $|$, denotes a septum which separates adjacent cells; the subscripts 1, 2, . . . associated with this symbol indicate the successive ages (or states) of the septum (number of timesteps which have elapsed since its formation). After the subscript has attained a particular value (in Fig. 11.2, this subscript value is 6 in accordance with the passage of i=6 timesteps), subscripts are omitted and the septa are considered mature. As shown in Fig. 11.2, at timestep i=1, the result of the division of cell $\vec{v}_2$ (which initiates the cellular descent) is a pair of daughter cells ($\vec{v}_2|_1\vec{v}_1$). At timestep i=2, the cellular trio ($\vec{v}_1|_1\vec{v}_2|_2\vec{v}_2$) has formed and this leads, at the next timestep i=3, to a filament of five cells which includes the v_1 cell destined to become a proheterocyst (bold in Fig. 11.2).

Taking into account the observed frequency of heterocysts along a filament of *A. catenula* in the context of a 2:1 *v* cell birth-length ratio, we suggest that, after i=3 timesteps, septum $|_3$ provides an appropriate condition for the determination of a proheterocyst two timesteps later. The v_1 cell which holds the $|_3$ wall in common with a v_2 cell of opposing polarity (i.e. $\vec{v}_1|_3\vec{v}_2$) undergoes one more division to give, at i=5, a pair of daughters. The resulting daughter, now a proheterocyst, *h*, exits from the cell division cycle and becomes quiescent. The cellular sequence in Fig. 11.2 shows that this cell has one septum $|_1$ and another at state $|_5$. When the septa at each end of a cell hold states $|_3$ and $|$, the proheterocyst *h* differentiates as a heterocyst *H*. A similar scenario, but with different age factors, applies in the case of filaments and their *H* cell differentiation, in which a 5:4 division rate differential applies to the daughter cells, as shown in Fig. 11.1.

In Fig. 11.2, all heterocysts begin to develop on the left side of septum $|_5$. Pursuing a hypothesis that cell isolation due to age-related transformations in the septum (see Giddings and Staehelin 1978) is a condition for *H* cell formation, it might also be possible for an *H* cell to appear on the right side of $|_5$. Thus, the polarity of the filament itself may provide yet another factor which decides heterocyst distribution: a left-to-right polarity could favour single *H* cells (as in Fig. 11.2) whereas an absence of such a polarity might favour the formation of pairs of *H* cells, as occurs in *Anabaenopsis arnoldii* (see Geitler 1960).

Rhythmic production of *H* cells is therefore a natural development emerging from the spatiotemporal pattern of division and septum maturation and closure. As mentioned above, a similar principle is hypothesised to apply within the cellular files of higher plants in circumstances where large and small daughter cells are produced by an unequal mother cell division (Barlow and Lück, unpublished data). One example is found in the epidermal cell files of *Arabidopsis* roots where the rhythmically developed hair cells become symplasmically isolated from their genealogically related non-hair neighbours (Duckett et al. 1994). Another example is the symplasmic isolation of stomatal guard cells from their neighbouring cells (Palevitz and Hepler 1985), even though some of these latter cells are related to the guard cells, being their sister or cousin cells.

11.4 Organogenetic Rhythms

We suppose that the relatively simple example of recurrent cell division and wall development in *Anabaena* is, in the final analysis, directed towards the production of heterocysts and, perhaps, also to spore formation. Now follows an example, from a higher plant, of a rhythmic organ production which has much the same 'goal'. Theoretical devices (L-system or Petri net) will be called upon to help illuminate the process.

Because of the physical restraints associated with the relatively stiff boundaries of higher-plant tissues and organs, the cells contained within these units are subject to compression. Consequently, they assume various geometric forms, resembling soap bubbles within a foam (Dormer 1980). Further, because every wall within this cellular 'foam' is the product of a cytokinesis, every cell bears a set of walls, each one of which has a different age. The two sister cells of a cytokinesis share the youngest wall (the division wall). It appears that, in natural cellular patterns, walls formed at cell generation *n* serve as a cue for the positioning of new division walls and, hence, to the pattern of walls at cell generation *n*+1, and so on. Plants thus seem to regulate the insertion of their walls in the interests of gaining an efficient control over organogenesis and histogenesis. Sometimes these patterns are absolutely deterministic, e.g. the cellular construction of adventitious roots of *Azolla pinnata* (Gunning et al. 1978) and *Ceratopteris richardii* (Hou and Hill 2004), whereas in other cases there may also be a degree of what appears to be indeterminacy (Barlow 1991, Barlow et al. 2001).

The age-relationship of the walls around a mother cell permits the patterns of previous cell-wall productions to be deduced. This gives the necessary information for the development of an L-system by means of which future cell productions can be predicted. Edges of cells are assigned rules for their extension and also bear information as to which of these edges will receive a new division wall at cytokinesis (Korn and Spalding 1973). Division rules

thus devised conform to map or M0L-systems (J. Lück and Lück 1985). Although the cellular arrays resulting from map L-systems are often drawn in two dimensions, other systems (stereomap L-systems) make it possible to give the array a certain amount of three-dimensionality (J. Lück and Lück 1985).

11.4.1 Angiosperm Shoot Apices and Their Phyllotaxies

In principle, there would seem to be no reason why the generation of axillary buds within the shoots of angiosperms should differ in any fundamental way from the shoot branching systems of cryptogams. The latter have already been modelled using an M0L-system (Barlow et al. 2001). We suppose that the processes regulating the formation and distribution of organogenetic centres (from which are derived both leaves and the associated adaxial buds within which are located new apical meristems) are due to a directed production of new cells via a rule-based generation of new cell walls. Organogenetic centres thus develop in accordance with a deterministic succession of cell-wall states and cell types.

The real-time aspect of shoot organogenesis is the phyllochron, the time between the formation of successive leaves of a specified size. The process is coupled to a mitotic clock and a set of cell growth-rate parameters, both of which regulate the rate of increase of the apical dome. During each phyllochron, the distal portion of the dome produces enough cells to ensure its own renewal, as well as acquiring (as a consequence of cell divisions) a set of wall states which permits the establishment of new organogenetic centres.

An algorithm (*A*3) was devised by J. Lück and Lück (1985) and by Lück and J. Lück (1993) to explore the potential of a superficial group of apical cells for bud and leaf inception. The outcome was the maintenance of the apex together with the rhythmic development of organogenetic centres and leaf primordia which were distributed upon the parastichies of two sets of Fibonacci spirals. In the natural state, bud and leaf productions are the result of events which involve the epidermal cell layer. For the purpose of modelling apical development, we take it as axiomatic that growth and division of the epidermal cells is coordinated with similar processes in the sub-epidermal cells which lie below the apex. Cells are referred to as either 3-, 4- or 5-sided, these numbers referring to the sides seen in surface view.

From a single 5-sided cell bearing the initial word ω_0=(1.2.3.4.5), algorithm *A*3 expresses the following state transition rules for cell edge production:

$$
\begin{array}{ll}
1 \rightarrow 3 & 6 \rightarrow 9 \\
2 \rightarrow 4 & 7 \rightarrow 0/_1 2 \\
2 \rightarrow 5/_6 7.8 & 8 \rightarrow 3 \qquad A3 \\
4 \rightarrow 9 & 9 \rightarrow 4.5/_6 7 \\
5 \rightarrow 0/_1 2 & 0 \rightarrow 8
\end{array}
$$

The *a* cell which commences the descent, with walls 1.2.3.4.5 (Fig. 11.3a), gives rise to a new *a* cell (a case of autoreproduction, although now the new cell has a different orientation to the mother *a* cell) and a sister cell *b* with walls 6.7.8.9.0 (Fig. 11.3b), and thence to a cell belonging to a cluster of three-sided cells (Fig. 11.3c). In the transition rules of *A*3, the solidus and its index, $/_i$, indicate the division wall and its label (for further details, see Lück and J. Lück 1993). Application of the rules over i=5 timesteps, in which all transformations are simultaneous, leads to the production of 64 cells. These cells comprise six merophytes which wind counter-clockwise towards the centre of the cellular array (Fig. 11.3d). From them emerge three spirals of leaves. Two alternative spirals can be seen to wind clockwise. The two sets of spirals thus conform to a 2/3 Fibonacci phyllotactic system.

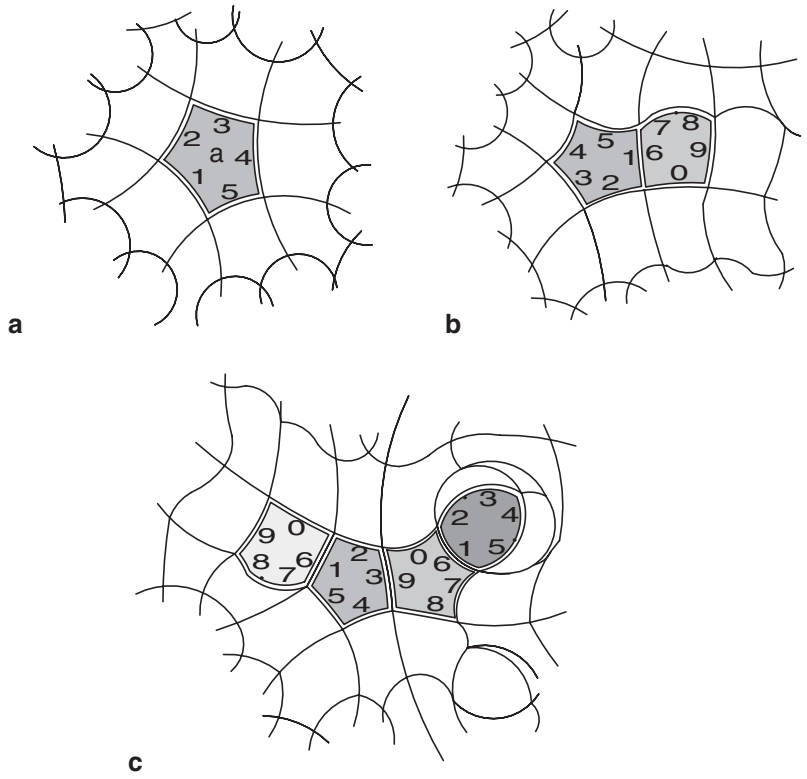

Fig. 11.3 Development of a shoot apex and autoreproductive centres after application of an L-system, represented by algorithm *A*3. **a** Five-sided *a* cell (*shaded*) at the centre of a shoot apex is surrounded by other, already formed cells. The walls of *a* are labelled 1.2.3.4.5, according to L-system notation. **b** After i=1 timestep, a new 5-sided *a* cell and a 4-sided sister cell, *b*, with walls 6.7.8.9.0, results from the first division. **c** After i=2 timesteps, new cells lie on either side of the original *a* cell. To the left is one 4-sided cell; to the right is a cluster of 3-sided cells protruding from the cell layer.

(*Continued*)

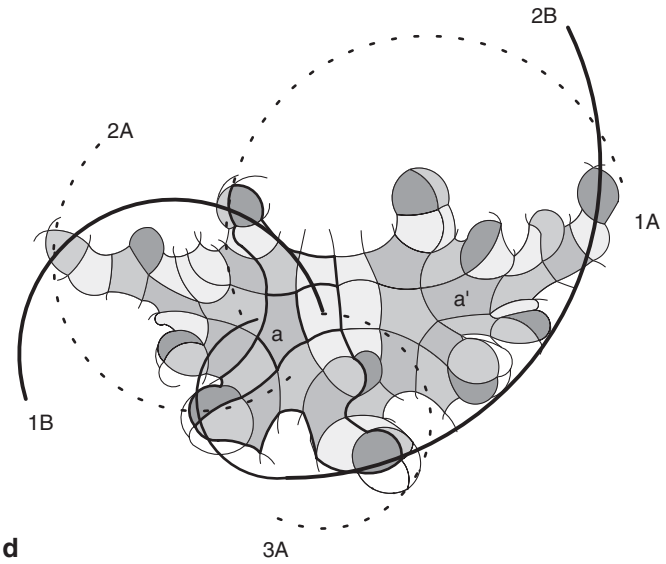

Fig. 11.3 (*Continued*) **d** At *i*=6, there are 64 cells developed within six merophytes; on the right of *a*, a unique cell signals the commencement of merophyte 6. The *a′* cell in merophyte 1 is a new initial cell (a 5-sided *a* cell) of a lateral meristem. Associated with this new, potentially organogenetic centre is a leaf-like structure which projects upwards; it is a composite structure formed from some cells of merophyte 1 and from other cells in two older, neighbouring merophytes. Every new merophyte traverses the same developmental steps. Spiral parastichies for the developing merophytes and organogenetic centres are indicated. Three *A* parastichies wind clockwise towards the centre of the apex, whereas two *B* parastichies wind counter-clockwise. The *A* and *B* sets of parastichies identify a 2/3 Fibonacci phyllotaxy (adapted from Lück and J. Lück 1993)

A third dimension can be added to the otherwise two-dimensional epidermal cell plate by letting it grow in conformity with a topographical map having defined contours. This procedure enables certain cells to push upwards from the apex (Fig. 11.3b, c). The upward growths co-locate with developing organogenetic centres, these being new and, at this stage, barely perceptible shoot apices, each accompanied by a more prominent, rapidly extending leaf primordium. Because they arise regularly upon each of the spirals (or helices, when the third dimension is considered) of merophytes, the spacing of these centres accords to a 2/3 Fibonacci phyllotaxy (Fig. 11.3d). This simulated shoot apex may therefore be regarded as an autoreproductive construction. It is the manifestation of a repetitive sequence of cell-based state transformations.

In natural shoot apical systems, axillary bud and leaf formation are accompanied by the extension of the lower portion of the apical dome, which becomes the internode. The growth of this internodal segment is under the regulation of various genes (Greb et al. 2003). Interestingly, however, in

seedlings of the rubber tree, *Hevea brasiliensis*, there can be rhythmic alternations of internode extension and non-extension, thus giving the shoot a 'lampbrush' growth form (Hallé and Martin 1968). Rhythms of shoot growth and their relationship to environmental factors have been reviewed by Borchert (1991).

Whereas algorithm *A*3 deals with 5- and 4-sided cells in which no offsetting of neighbouring division walls is permitted, 'real' shoot apical cells have a different numerical distribution of edges. As has been exhaustively catalogued (Dormer 1980), within two-dimensional cellular arrays the modal number of wall–wall contacts is six. The extra walls come into existence as a consequence of the offsetting of neighbouring division walls at cytokinesis and the interpolation of short α segments. These segments then extend and become a de facto wall. Nevertheless, 4- and 5-sided cells might be considered to be the natural cellular polygons, any supernumerary walls arising in a secondary manner. In the present context of organogenesis, the question whether α segments influence the phyllotactic spiral by means of the orientation of the cellular tetrads which they construct remains to be explored. Recalling the simple branching systems in cyanobacteria, an interesting example is found in *Stigonema mamillosum* where, according to Golubić (1967) and also illustrated by Geitler (1960), lateral branches grow out from a parental filament in regular whorls and helices which resemble higher-plant phyllotaxy. These helical branch arrangements may be due to an effect, expressed within the branch initials, of an offsetting of new division walls with respect to those already present in the preceding merophyte.

11.4.2 The Plastochron

Often confused with the time between the initiation of successive leaves, the plastochron, as originally proposed by E. Askenasy in 1880 (Erickson 1959), refers to the time (*Formungszeit*) between the initiation of two successive internodes. Later, in 1897, K. Giesenhagen made observations upon growing shoots of the green alga *Nitella flexilis* (Erickson 1959). Here, he saw that the production of each node and internode was accompanied by the division of the single shoot apical cell. In fact, in this system, a pair of asymmetric transverse divisions is crucial for the recognition of each plastochron: the apical cell (*a*) divides unequally to regenerate itself as an *a* cell and to produce a smaller proximal daughter cell (*b*). Cell *b* then divides to give a distal cell, b_a, of which the products form the node. The proximal cell, b_b, does not divide and proceeds to form the internode. Node and internode are structural elements of a phytomer, a rhythmically repeating morphological unit characteristic of modular plant organisms. In the case of *Nitella*, because the cells of each phytomer trace to a single sub-apical cell, *b*, the phytomer is also equivalent to a merophyte. (A merophyte is comprised of a *b* cell and its descendents, which have been derived from an *a* cell.) However, in plants with larger

shoots and multicellular apical domes, many merophytes (or cell packets) may be included within each phytomer.

Analogously to *Nitella* and many other algae, the shoots of angiosperms show a relationship between the plastochron and the rate of enlargement of the terminal portion of the shoot apex. Rather than a single cell being involved (as in *Nitella*), it is a sub-distal portion of the multicellular apical dome which contributes to the phytomer. Each phytomer consists of an internode, a node plus associated leaf and axillary bud, and sometimes one or more root primordia (Barlow and Palma 1997). During steady-state growth, the time taken for the production of one of these units is the same as that required for one doubling of the apical dome. Thus, successive plastochrons relate to the rhythmic transformations of the apical dome with respect to the number of its cells and its volume, and at the same time the dome renews itself. In addition, the dome delivers cohorts of cells (merophytes) into each nascent phytomer with its new organogenetic centre. The intrinsic conditions which govern the rhythm of the dome are those which constitute an organogenetic, plastochronic timekeeper.

11.4.3 A Petri Net Representation of the Plastochron

Studies of the genetics of *Arabidopsis* development have revealed that the genes *WUSCHEL* (*WUS*) and *CLAVATA* (*CLV*) are central to the regulation of shoot meristem activity and the form of the shoot apex. The interactions of these two genes, and of others (e.g. *POLTERGEIST*, *SHEPHERD*, etc.) which facilitate their function, have been described in numerous authoritative reviews and research articles (e.g. Clark et al. 1996; Schoof et al. 2000; Sharma and Fletcher 2002; Carles and Fletcher 2003; Lenhard and Laux 2003). Our hypothesis is that the interrelationship between *WUS* and *CLV* is connected with phytomer production and the plastochron. Therefore, in order to uncover something more about the workings of these intricate states and processes, a Petri net was constructed (Fig. 11.4). Occurrence graphs (Fig. 11.5) accompany the net and summarise the sequence of places and state transitions.

Three *CLV* genes cooperate to maintain a population of stem cells, S, located at the summit of the shoot. Descendents of the *CLV*-supported S cells at the summit of the apex enter a pool of sub-apical meristematic cells, M, where their numbers are amplified over the course of a plastochron. Eventually, they produce new buds, nodes and internodes, as mentioned above. Plants which are mutants of *CLV* are able to grow but do so in a peculiar way. Due to the mutation, the normal, isotropically growing apical dome is transformed into an anisotropic fasciated apex which has the appearance of a crinkled ribbon (Clark et al. 1993). Mutant apices can, perhaps, be understood as having lost the ability not only to orient the output of cells from the apex along the usual parastichies (cf. Fig. 11.3) but also to organise the cells

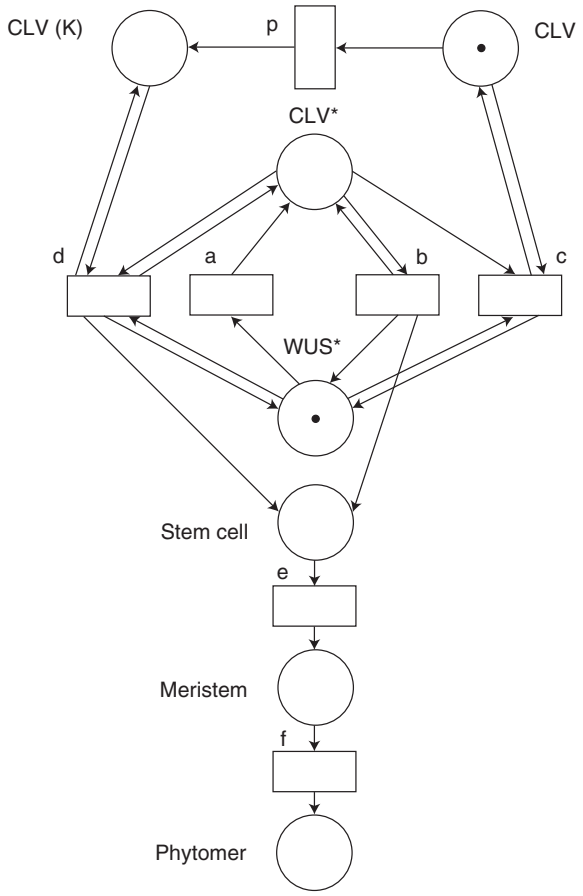

Fig. 11.4 A Petri net which shows the changing states of *WUSCHEL* (*WUS*) and *CLAVATA* (*CLV*) gene expression domains in the shoot apex of *Arabidopsis thaliana*. The net illustrates both the wild-type situation and findings of the knock-down experiment of Reddy and Meyerowitz (2005), in which *CLV3* is silenced by a glucocorticoid-induced RNAi. *Circles* (places) represent states. The upper two places, CLV and CLV(K), represent the wild-type *CLV3* gene and the knocked-down version of *CLV3*, respectively. The inductive knock-down event enables place CLV to assume immediately its alternative form, CLV(K). The two central places, WUS* and CLV*, represent the expression domains of these genes (or gene complex, in the case of CLV) within the shoot apex. The lower three places represent stem cells, meristem and phytomer, the cells of which pass in succession through these compartments as the apex grows. *Rectangles* are enabling transitions: they permit transition from one state to another, according to the figurative convention for Petri nets (for further details, see text). Information on gene interactions and the *CLV*-knock-down experiment are described in the text and in the cited original articles. Outcomes of the net interactions are shown in the two occurrence graphs in Fig. 11.5. The interactions are hypothesised to be related to the plastochron and also, in ways not understood, to the fasciated form of the shoot apex which is characteristic of both the mutated and the knocked-down states of the *CLV* genes. When places are marked with a token, they are considered to be active. Here, the net is marked (*solid circles* within two of the places, CLV and WUS*) as it might be early in shoot growth. The place 'Phytomer' is able to accept multiple tokens

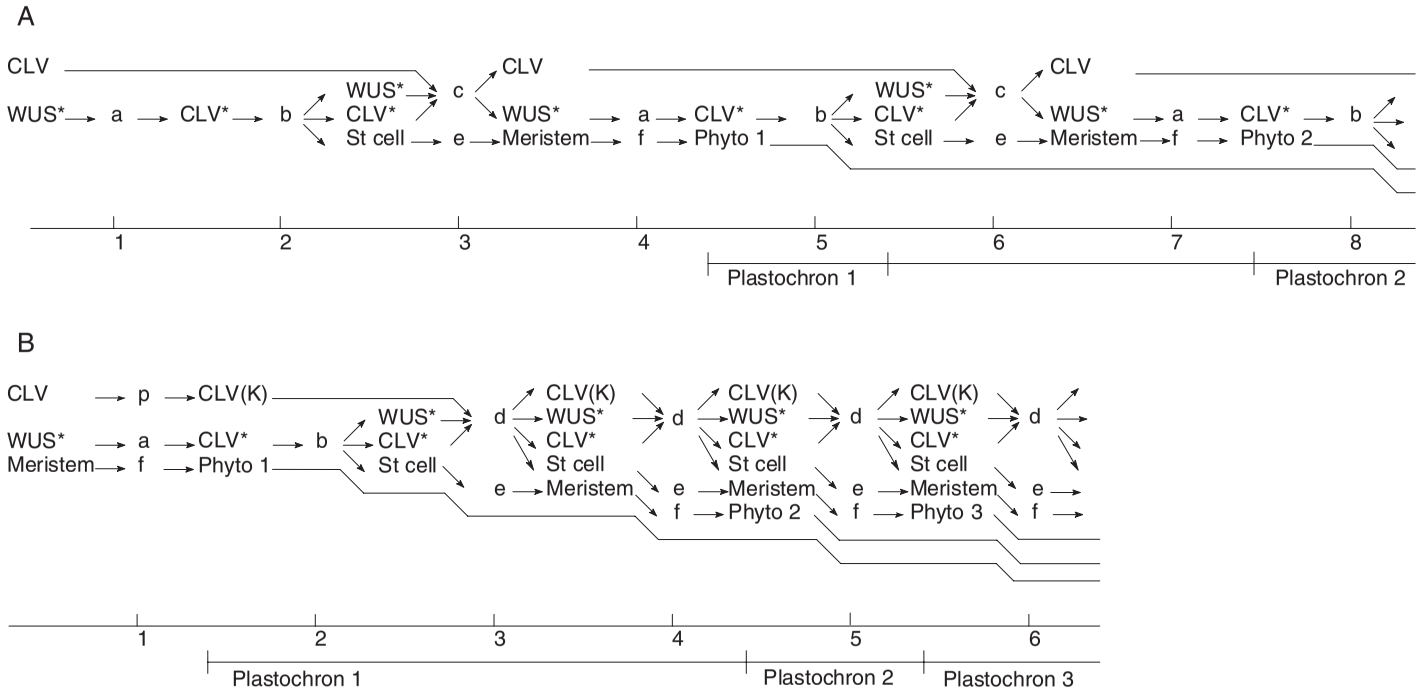

Fig. 11.5 Occurrence graphs of place transformations relating to the Petri net of Fig. 11.4. The horizontal scales indicate the succession of transitions (these transitions are represented by *rectangles* in Fig. 11.4). Below each of these scales is a second scale depicting the duration of the plastochron. Each plastochron commences when a marker is added to (and accumulates in) the place 'Phytomer' in Fig. 11.4. **a** Graph depicting progress of the net following its initial marking, as shown in Fig. 11.4. It corresponds to a wild-type *Arabidopsis* shoot apex. There is a regular gain and loss of marking of the place CLV^*. St cell denotes stem cell. **b** Graph depicting the switch, when event *p* is enabled at transition 1, whereby the knocked-down state of *CLV* (place CLV(K)), is reached due to the induction of RNAi. Then commences oscillation between the two states, WUS^* and CLV^*. As a consequence, stem-cell activation is continual, and it may be this which leads to the fasciated form of the apex. Plastochrons are now of shorter duration than in wild type. This is consistent with the observed accelerated rate of cell production in the apices of knocked-down *CLV* plants (Reddy and Meyerowitz 2005)

into a vertical succession of phytomers. An L-system algorithm permits the theoretical development of the fasciated apex (J. Lück, unpublished data). The fasciation does not arise simply because the apex is excessively large. *Arabidopsis* seedlings mutant for the gene *ULTRAPETALA* have enlarged apices which are not fasciated.

Young *Arabidopsis* seedlings mutant for *WUS* show premature termination of shoot apical meristem activity. *WUS* is therefore inferred to maintain *CLV* and, thereby, to activate stem-cell division. Accordingly, in the *wus* apex, the S cells as well as their descendents, M, cease division, having become prematurely committed to some form of differentiation. The *wus* mutant therefore seems to regulate the organisation of its shoot apex in a way which contrasts strongly with the effects of the *CLV* mutants with their fasciated apices.

The extent of the cellular domain in which *WUS* is expressed is also regulated by *CLV*. In *clv* mutants, excessive widening of both the *WUS* and the overlying *CLV* expression zones accompanies the fasciation of their apices (Brand et al. 2000). Nevertheless, although fasciated, enlarged vegetative shoot apices are still able to form leaf primordia. The inference is that the CLV protein restricts the activity of the *WUS* gene, whereas *WUS* promotes functionality of the *CLV* gene complex. Together, the two genes secure steady-state cell production at the apex. Thus, there appears to be a feedback between *CLV* and *WUS*, and this feedback is expressed also at the level of the respective cellular domains in which *WUS* and *CLV* operate. These two domains retain a particular range of sizes even though they fluctuate periodically in their actual extents. The rhythmic on–off activity of the *WUS* domain has recently been modelled in terms of diffusion-reaction kinetics by Jönsson et al. (2005). The same type of kinetic system has also been proposed to control the rhythmic spacing of *Anabaena* heterocyst development (Wilcox et al. 1973).

Cytological observations indicate that the plastochron might be related to the rhythmic expansion and contraction of the domain in which *WUS* is expressed, due to that gene's interaction with *CLV*. The *WUS* domain appears to coincide with the 'central zone', CZ, of the angiosperm shoot apex which, as conventional cytological methods (staining, counting mitoses, etc.) have revealed, fluctuates in size during a plastochron (Nougarède 1967). The dynamic activity of CZ probably correlates with its rates or rhythms of cell production and the accompanying passage of cells into the proximal portion of the meristem (M) and, thence, into the sub-apical zone where the cells lose their ability to divide. In this last-mentioned zone, cell determination leads to the differentiation of successive phytomers which are added to the elongating shoot. We conjecture, therefore, that the *WUS-CLV* feedback interaction, as shown in the Petri net of Fig. 11.4, is a representation of the plastochron and, hence, constitutes a timer of phytomer production. The occurrence graph in Fig. 11.5a provides an account of the steps in this interaction, and how they relate to successive plastochrons and to phytomer formation.

As commented by Reddy and Meyerowitz (2005), analysis of the known mutants which affect apical growth in *Arabidopsis* may be inadequate to

define precisely the function of the respective genes within the network of interacting genes. This is because loss or gain of a gene function affects the entire gene network, especially when the developmental timescales are relatively long, a circumstance which may permit the gene network pathways as well as the network of communicating cells to make compensatory rearrangements. With this possibility in mind, these authors constructed a transgenic plant system in which the *CLV3* gene could be silenced, or 'knocked down' (*kd*), by a constitutively activated, interfering double-stranded RNA molecule (RNAi; Reddy and Meyerowitz 2005). The *kdCLV3* condition should also be reversible, thereby restoring normal activity of the gene (Doerner 2006). The rapid activation of the RNAi enables the earliest effects of a silenced *CLV3* gene upon the apex to be observed. The Petri net (Fig. 11.4) accommodates the *kdCLV* gene condition. A supplementary event, p, has been introduced into the net so that wild-type place CLV can be switched (by activation of the knock-down RNAi construct) to the alternative *kd* form, CLV(K), hence promoting the development of what is effectively a phenocopy of the *clv3* mutant.

When CLV(K) impinges upon the net (Fig. 11.4), the domains of *WUS* and *CLV* show continual, uninterrupted cycling. Microscopic observation of the knock-down apices indicates that the *WUS* and *CLV* domains show coordinated growth but lack the normal regulation of their dimensions. There is also a continual passage of stem cells, S, into the M compartment of the dome. As indicated by the occurrence graph of Fig. 11.5a, in the wild-type apex there are breaks in the *WUS-CLV* cycle which we consider to define the limits of the plastochrons, and these are accompanied by phytomer productions. The breaks in the cycle coincide with the periodic activation of the S cells. By contrast, in the knocked-down state (Fig. 11.5b), activation of the S cells is continuous and is accompanied by the absence of obvious phytomers – i.e. it is similar to the fasciated condition, in which the dome becomes as wide as it is in the *clv3* mutant.

However, the situation is probably more complex than indicated. For example, there are genes which regulate meristem size. Analysis of one of these, *HANABA TARANU*, indicates that signals are sent to the stem cells at the summit of the apex from cells located at the transition between meristem (M) and differentiation zone (D) (Zhao et al. 2004). So, the rhythm of the plastochron may feed back upon the rhythm of stem-cell activity.

11.4.4 Rhythms of Cell Determination and the Plastochron

The determination of cells for both the node and the internode portions of the phytomers may come about in much the same way as we proposed for the radial patterning of cell types in secondary phloem (see Barlow and Lück 2005, 2006), i.e. the control resides in an interaction between the relative rates of cell displacement through the proximal part of the apical dome and a gradient of morphogen. The differential rate of passage of cells through the zone

of determination leads them to experience various cumulative levels of morphogen which then permit different thresholds to be crossed, thereby triggering specific determination events, such as whether cells should elongate (internode) or not (node). One spectacularly visible bifurcation of node–internode development relates to whether certain idioblast cells undergo cytokinesis following mitosis. In *Sambucus nigra*, the absence of cytokinesis is a feature of single cells in the distal part of idioblast cell complexes: such cells are located in the node and become multinucleate and polyploid. The presence of cytokinesis is a feature of the cell in the proximal portion of the complexes: such cells are located in the internode where they divide, their descendents remaining diploid (Zobel 1985).

The stem-cell zone of root apices – the quiescent centre (QC) – goes through phases of expansion and shrinkage. However, when root apices of tomato with the *gib-1* mutation (which severely depresses gibberellin biosynthesis) are grown in liquid culture, their quiescent centre shows continuous enlargement (Barlow 1992). Apparently, these mutant roots are unable to regulate QC dimensions. Here, perhaps, there is some similarity with the situation in the shoot of the *clv* mutants of *Arabidopsis* where both the CZ and the domain of *WUS* gene expression are enlarged (Clark 2001). Similarities between shoot and root apices with regard to their stem-cell activities have been reviewed by Veit (2004) and by Vernoux and Benfey (2005). Moreover, it is not improbable that the development of diaphragms within the cortex of *Thalassia* roots (Tomlinson 1969) has some connection with a cyclic activity of the quiescent centre from which the cortical cells are derived (Barlow and Lück, unpublished data). The short diaphragm cells and the longer intervening cells have at least a parallel, if not a homology, with the generally short nodal cells and the longer internodal cells of the shoot, respectively. Thus, each diaphragm marks the passage of a time unit in the form of a 'root plastochron' which traces back to a rhythm of cellular (and maybe genetic) activity in the root's stem-cell zone.

11.5 The Cycle of Life

Sexually reproducing plants show one ultimate oscillation with respect to their morphological states – the alternation of the sporophyte and gametophyte generations. In terms of a Petri net (see Fig. 11.6), the oscillation between places (generations) of the net depends upon two self-maintaining places. In nature, one of these places is represented by any ubiquitous pollinator (wind, insects) which effects the link between generations. The other place is a condition found in the environment, often in the form of daylength requirement for flowering. Although performing a vital function in the oscillatory cycle, the existence of the perennial pollinator also intimates of its wider perspectives – the other interconnected nets of which the ecosystem, with its multitude of life

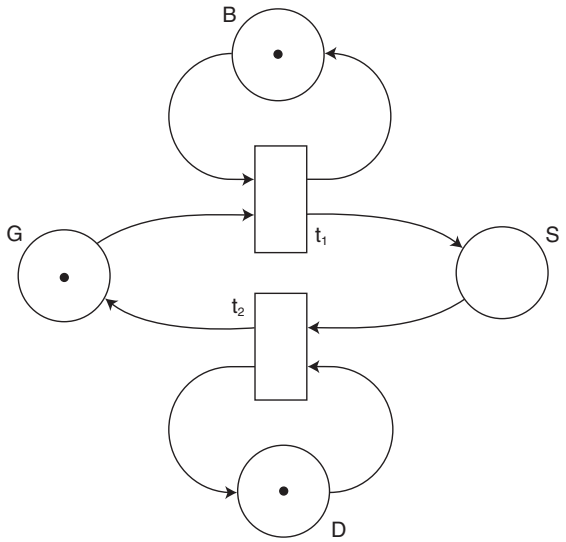

Fig. 11.6 Petri net which expresses the alternation of the gametophyte (place G) and sporophyte (place S) generations of plants (pteridophytes, gymnosperms and angiosperms). t_1, the G-to-S transition, is dependent upon water or, in some angiosperms, a living pollinator such as an insect. These are collectively represented by place B. t_2, the S-to-G transition, is dependent, in some angiosperms at least, upon an environmental signal (e.g. daylength), place D. The initial marking of the net is at places B, D and G

forms, is comprised. By contrast, daylength remains fixed. These two conditions nicely illustrate the dialectic of nature – the cooperation between hazard and determinism.

11.6 A Glimpse of Cell Biology and Morphogenetic Rhythms

Vibrations, rhythms and oscillations are principles of the created world in both its visible and invisible aspects. Particles of matter from which life was fashioned originally moved randomly, free from constraints, in accordance with Brownian motion. When such particles began to cohere, however, their innate movements became more restricted and motion was no longer random. In the course of time, a moving material point might have returned to its original position: it would then have exhibited oscillatory behaviour. The possibility for directed movement then began to be regulated and, for this, structures were required which would direct (and restrict) such movements.

At the level of the cell, oscillations of the constituent macromolecular elements participate in the building of tissues and organs. Dramatic evidence of

this is seen in cells without walls, which show rhythmic breaking and remaking of the external membrane – events which trigger rhythmic bursts of cellular movement (Paluch et al. 2006). A more subtle rhythm is displayed by the recurrent passage of cells through the mitotic cell cycle in accordance with the molecular oscillations of the mitotic clock. Then, when genes and cells are organised into suitable configurations, recurrent patterns of spatially directed mitotic behaviour in the multicellular arrays result in the recurrent creation of potential organogenetic zones. Thus, rhythms and oscillations have become the keys to the autoreproduction not only of new cells but also of new organs (branch roots, branch shoots, leaves); the timers of these rhythms are, in addition to the mitotic clock which regulates cell productions, the phyllochronic and plastochronic clocks. As may be evident from our chosen examples of L-systems in relation to recurrent developmental events, an understanding of biological rhythms and oscillations depends upon the discovery of a unique set of conditions or states which enables a system to return to one particular state which can be designated as the 'initial state', or 'start'. A 'timer' of a biological process becomes evident only when the conditions for recurrence are fulfilled once more.

In order to comprehend cell replication, it is necessary to pay attention to the reduplication and separation of the cell body (Baluška et al. 2004, 2006). The cell body is composed of a nucleus surrounded by a cytoplasm which houses a perinuclear microtubule-organising centre (Baluška et al. 1997). This latter component, besides being responsible for the construction of the mitotic spindle, is able also to direct the development of a 'peripheral apparatus'. Not only does this apparatus serve as a protective outer covering for the cell body but its two principal components, the plasma membrane and the underlying cortical cytoplasm, also cooperate to support an actin cytoskeleton (Collings and Wasteneys 2005). In addition, the peripheral apparatus of plants is the seat of sets of cortical microtubules and their organising centres, the latter probably being derived from the perinuclear organising centre (Baluška et al. 1997). The peripheral apparatus, moreover, maintains its own pattern of development which, with the assistance of the cortical microtubules, includes an ability to construct tissue-specific cellulosic coverings (or walls) for the composite ensemble of cell body plus peripheral apparatus.

Possibly, it is the cell body which Gilbert and Lloyd (2000) considered as being the candidate for the autodynamic multi-oscillator system which constitutes the fundamental unit of living matter. The key word here is the adjective 'autodynamic', for whereas oscillating chemical or biochemical systems are products of differential reaction rates, their oscillations eventually decay; by contrast, reactions which occur in open systems far from equilibrium lead to *sustained* oscillations. Evidence for this is seen in non-biological, chemical systems (e.g. the Beloussov-Zhabotinsky reaction), and in cell extracts (assayed for glycolysis, for example) where a periodicity of reaction cannot be sustained. Could it be that the autodynamism of the 'living' reactions, out of

which sustainable biological oscillations arise, depends upon a particular structuring of the cytoplasmic space (at the cellular level), and even upon the spatial disposition (at the organismic level) of the cells themselves, as in the case of acrasin-induced rhythmic aggregation of *Dictyostelium* amoebae (Kessin 2001)? Furthermore, could it be that autodynamism is assisted by the movements of reactants from one discrete reaction site in the cytoplasm to another, and that these movements conform more to the properties of molecular motors than to the laws of diffusion, important as these latter may be at the atomic or molecular scale? These vital questions were discussed by Aon and Cortassa (1997) in connection with the relationship between cellular metabolism and cytoplasmic structure. The authors concluded that the dynamics of biochemical reactions, when integrated with the rheology of a polymeric cytoplasmic network, as well as with the self-organising properties of nonlinear kinetic systems, lead to new spatiotemporal behaviours such as oscillations, thresholds, and chemical waves. Crucial in reaching this view is the property of 'dynamic instability' (Kirschner and Mitchison 1986), which governs the continual and self-sustained assembly and disassembly of microtubules. It is an oscillation which permits the inherent tendency of microtubular self-assembly to be put at the service of morphogenesis.

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12 Molecular Aspects of the *Arabidopsis* Circadian Clock

Tracey Ann Cuin

Abstract

Circadian rhythms are endogenous, self-sustaining and self-generating cycles of an approximately 24-h period that are present in all living systems that prepare organisms for periodic environmental changes of light and temperature. Although poor compared to animal systems, our understanding of the circadian system in plants has increased considerably in recent years. This review presents our current knowledge of the plant circadian system, focussing on that described for the model plant, *Arabidopsis thaliana*, upon which most of our information is based. The repertoire of proteins that play a crucial role in clock regulation, as well as the photoreceptors and signal-transduction pathways proposed to be involved in transmitting light information to synchronise the endogenous clock with the outside world are described. Finally, our current understanding of how the mechanisms by which the central oscillator controls the activity of the various clock controlled genes, the "hands of the clock", is briefly discussed.

12.1 Introduction

All organisms show a rhythm of metabolism and physiological responses in tune with the day/night cycle of the earth. Humans for example, similar to other diurnal mammals, have a greater rate of alertness, higher body temperature and blood pressure and increased kidney function during the day, with an increased propensity to sleep at night (Foster and Kreitzman 2005). In insects, the adult flies eclose from their pupae in synchrony with dawn to provide the maximum chances of survival (Pittendrigh 1954). Even cave-dwelling species of fish and insects retain the diurnal rhythms of their ancestors (Foster and Kreitzman 2005). In plants, regular rhythms of leaf movements have long been known and as far back as the 18th century, the endogenous nature of diurnal rhythms was established for leaf movements of *Mimosa pudica* (de Mairan

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1729). These phenomena are not purely in response to the external environment – they continue in the absence of exogenous cues. A plant maintained in constant light still shows cyclic production of its photosynthetic machinery (Millar and Kay 1996), a human deep underground continues to wake and sleep with a period close to, but not exactly 24 h (Luce 1971). Such processes are known as circadian rhythms (from the Latin *circa*; approximately, and *dies*; day). They are temperature compensated and self-sustaining, "free-running" with periods of about 24 h, the cycles being maintained from oscillations in the levels of certain molecules within the cell. The molecules making up this core clock and their biochemical interactions have been well studied and in insects and mammals, the components of this basic "clock mechanism" are well known. Conversely, although prior to 1900 plants were the primary system for studying circadian rhythms (e.g. Darwin and Darwin 1880), the mechanisms of the clock have remained more elusive. Only recently, with the development of robust, high-throughput assays for measuring rhythmic changes in promoter activity (Millar et al. 1992), has our knowledge of the circadian system in plants increased considerably. This review describes our current understanding of the *Arabidopsis thaliana* circadian system.

12.1.1 Defining Features of Circadian Rhythms

Three properties of diurnal rhythms form the diagnostic criteria of an endogenous circadian system:

1. Circadian rhythms are self-sustaining and self-generating, persisting under constant conditions when the organism is deprived of external time cues, with an approximate 24-h period.
2. The circadian clock is entrained by environmental stimuli.
3. The free-running period of circadian rhythms is temperature compensated.

12.1.2 Overview of the Circadian System in *Arabidopsis*

Classically, the circadian clock has been divided into three components (Fig. 12.1). The first; the "input" pathway, is how external cues "entrain" the second component; the endogenous oscillator, to the environment. This initiates the "outputs", the third component, which are the overt rhythms under the control of the circadian system.

However, this traditional three-component model of the circadian system is overly simplistic and a far more complicated picture is emerging, with output elements modulating the pace of the oscillator and input elements themselves being tightly controlled by the clock. The presence of multiple input receptors and output pathways results in a further escalation in the complexity of the model. Finally, there may even be multiple clocks operating both within the

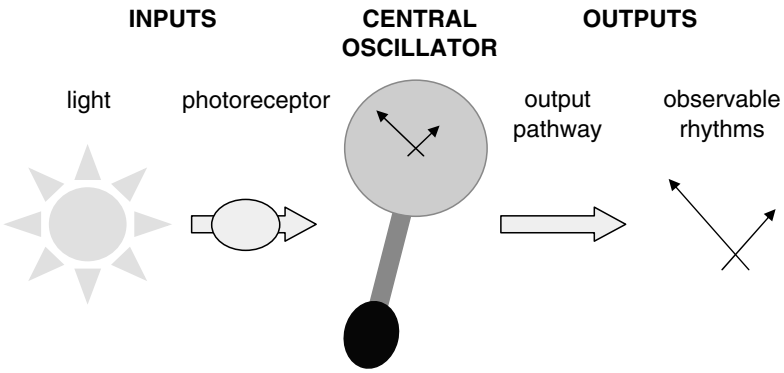

Fig. 12.1 Organisation of the circadian system: a simple model for the major components. Light information is perceived by a photoreceptor that passes the information along to the central oscillator, entraining it to the correct time of day. This core oscillator, via the output pathway controls the observable rhythms

same organism and possibly even within the same cell. Thus, the simple linear model of Fig. 12.1 is inadequate and a more complex model has been proposed as shown in Fig. 12.2. Despite the increasingly intricate picture of the circadian clock model, this review will describe each of the basic components in turn; the inputs into the clock, the central oscillator and finally, the outputs of the clock.

12.2 Entrainment – Inputs to the Clock

One of the diagnostic features of circadian rhythms is that they persist in the absence of external cues. However, the deviation of the free-running period from 24 h means that an endogenous clock would rapidly fall out of phase with local time. Thus, the oscillator must be entrained with the environment. For plants, the changes in light and temperature encountered at dawn and dusk serve as the chief entrainment cues: the "zeitgebers". Entrainment is achieved by effecting a stable change in the phase through the modification of mRNA, protein and/or activity levels of one or more oscillator component. This change in phase does not alter the internal sequence of processes, but re-aligns it with the daily environmental progression, enabling the organism to adjust to changing day-length during seasonal transitions (Devlin 2002).

12.2.1 Light

Circadian clocks, without exception, respond to light (Roenneberg and Foster 1997) and light is the best-characterised entraining stimulus in plants (Devlin 2002; Somers 2005). Correct entrainment is dependent (in part) on different

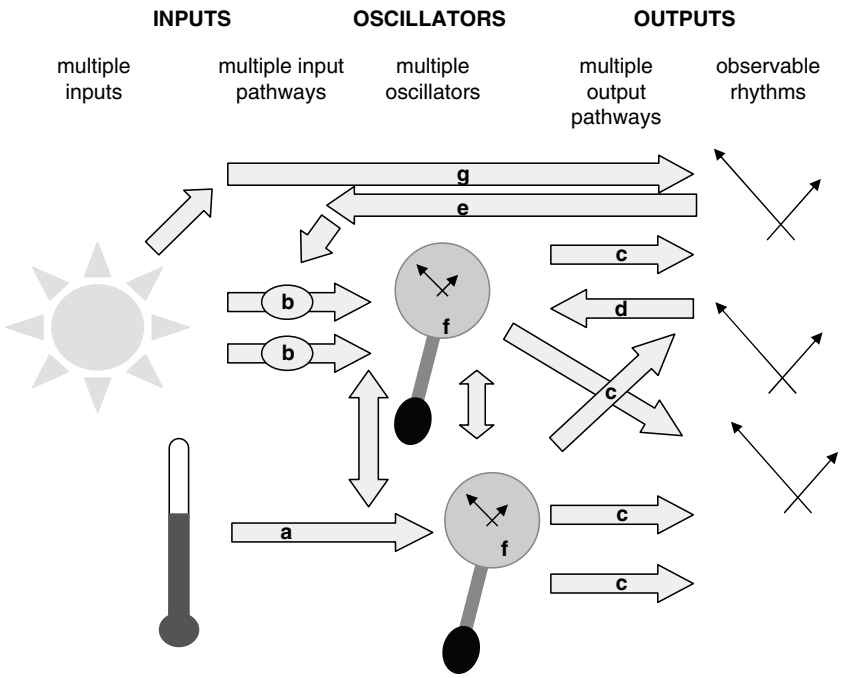

Fig. 12.2 The circadian system is much more complex than that shown in Fig. 12.1. Temperature entrainment (*a*) is involved, in addition to light. There are also multiple photoreceptors and input pathways (*b*), and output pathways (*c*). Certain outputs may feed directly back to the central oscillator (*d*) and the input pathways (*e*). There may even be multiple oscillators that can interact with each other (*f*). In addition to control by the circadian system, the entraining stimuli may be controlled by external stimuli (*g*)

responses to light during the cycle – the response is "gated". The mechanisms by which gating occurs are obscure, but provide evidence for the oscillator being responsive to resetting cues only at appropriate times (Somers 2005). The light signal must also be relatively prolonged (Devlin 2002), thus preventing resetting by inappropriate signals, such as moonlight or lightning.

Three main families of photoreceptors are present in plants. These are the red and far-red light-absorbing phytochromes (Quail 2002) and the blue and UV light-absorbing cryptochromes and phototropins (Whitelam and Devlin 1998; Christie and Briggs 2001). The ZEITLUPE family of proteins may also detect blue/U-A light (Imaizumi et al. 2003) and there is evidence of a green light photoreceptor, although its nature is unknown (Folta 2004). Together, these photoreceptors track the changing intensity and spectrum of light; they pick up its direction and maybe even its plane of polarisation (Kendrick and Kronenberg 1994).

The *Arabidopsis* genome encodes five phytochromes: PHYA-PHYE (Sharrock and Quail 1989; Quail 2002; Nagy and Schäfer 2002). The cryp-

tochromes are part of a three-gene family in *Arabidopsis*; CRY 1-3 (Lin and Shalitin 2003), although CRY3 localises to the chloroplasts and mitochondria, so is unlikely to participate in photoentrainment (Kleine et al. 2003). Light-labile PHYA is the predominant photoreceptor at low intensities of red or blue light whereas PHYB dominates at high intensities of red light (Somers et al. 1998a). *phyD* and *phyE* mutants have clock phenotypes in red light, but only in the absence of PHYB, suggesting they act redundantly with PHYB (Devlin and Kay 2000; Devlin 2002). CRY1 also appears to be involved in red light signalling under low-fluence red light (Devlin and Kay 2000), although this may be downstream of PHYA in the signal transduction pathway (Devlin and Kay 2000; Devlin 2002). CRY1 dominates at high intensities of blue light (Somers et al. 1998a) whereas CRY2 acts at intermediate intensities of blue light, although redundantly with CRY1 (Devlin and Kay 2000; Somers 2005). Not all the red light input into the clock is accounted for by the action of the PHYA-E photoreceptors (Devlin and Kay 2000; Devlin 2002), implying the involvement of additional photoreceptors.

The phototropins in *Arabidopsis* are PHOT1 (Non-Phototropic Hypocotyl; Liscum and Briggs 1995), PHOT2 (Kagawa et al. 2001) and zeaxanthin (Christie and Briggs 2001). These photoreceptors are involved in the perception of blue wavelengths, leading to phototropism and stomatal responses (Kagawa et al. 2001), but evidence is lacking for a role in photoentrainment (Harmer et al. 2000; Millar 2003).

12.2.2 Pathways to the Central Oscillator

As outlined above, the plant circadian clock uses many photoreceptors that require several input pathways to the oscillator (Fig. 12.3). These pathways are currently unknown, but a number of proposals have been made in the literature.

12.2.2.1 The PIF3 Model of Light Signalling

The Phytochrome-Interacting Factor 3 (PIF3) is a DNA-binding protein of the bHLH family of transcription factors (Heim et al. 2003; Toledo-Ortiz et al. 2003). Upon illumination with red light, PHYB translocates to the nucleus (Nagy and Schäfer 2002), suggesting a very short signal transduction pathway between light perception and gene transcription (Quail 2002). In the nucleus, PHYB complexes with the nuclear-located PIF3 already bound to G-boxes (Ni et al. 1999). Such G-box sequences are present in many light-activated genes, including the clock-associated genes *CCA1* and *LHY* (Martínez-Garcia et al. 2000). PIF3 may bind to these, resulting in their induction (Makino et al. 2002; Yamashino et al. 2003).

As mutants with altered PIF3 function show altered light responses in vivo, this is clearly one potential mechanism of photoentrainment.

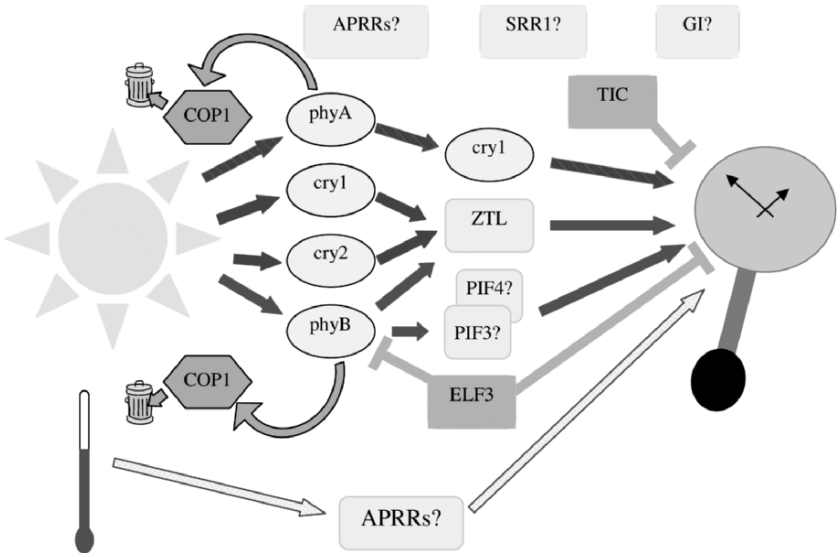

Fig. 12.3 Light input to the oscillator, via phytochromes and cryptochromes. The input is via phytochromes and cryptochromes. The input pathway is still uncertain. ZTL appears to be involved, although the function of SRR1 and GI is still uncertain. The involvement of PIF3 is now questioned, as are the APRRs, although the latter may play a role in temperature entrainment. TIC and ELF3 have a gating mechanism on the clock at dawn and evening respectively. COP1 interacts with PHYA and PHYB leading to their degradation

However, loss-of-function of *PIF3* does not affect period length (Monte et al. 2004) and lacks circadian defects (Yamashino et al. 2003; Salomé and McClung 2005a). Furthermore, there are suggestions that *PIF3* actually antagonises some light responses (J.Y. Kim et al. 2003; Bauer et al. 2004; Monte et al. 2004; Park et al. 2004).

Although the role of PIF3 acting as a positive factor in phytochrome signalling has been rejected, PIF3 does interact with PHYB in the nucleus (Bauer et al. 2004). Current evidence indicates that rather than being a component of the clock, PIF3 acts as either a positive or a negative regulator of phytochrome signalling, but not in photoentrainment (J.Y. Kim et al. 2003; Monte et al. 2004).

Other bHLH proteins closely related to PIF3 have also been implicated in phytochrome signalling (Toledo-Ortiz et al. 2003; Yamashino et al. 2003). PIF4 may play a role in PHYB-mediated red light signalling and HFR1 (Long Hypocotyl in Far-Red) in PHYA-mediated far-red light signalling (Huq and Quail 2002; Duek and Fankhauser 2003). PIL1 (PIF3-Like) and PIF4 are involved in shade avoidance and red light responses respectively (Huq and Quail 2002; Salter et al. 2003). Other identified members of this family include PIF5 and PIF6 (Fujimori et al. 2004; Oh et al. 2004). All PIF3-like family members, excepting HFR1 interact with TOC1 (Yamashino et al. 2003). However,

none of the bHLH proteins has a confirmed role in photoentrainment (Makino et al. 2002; Yamashino et al. 2003).

12.2.2.2 ZEITLUPE Family and Phototransduction

The *ZEITLUPE* (*ZTL*) genes possess three distinct domains: the LOV (Light Oxygen Voltage) domain, an F-box domain and kelch motifs. The LOV domain is a flavin-binding light-sensitive domain (Somers et al. 2000). The kelch domain is a protein-protein interaction motif, and along with the F-box, implicates these proteins in ubiquitin-mediated proteasomal degradation (W.Y. Kim et al. 2003). A related gene, the *Flavin-Binding Kelch Repeat F-Box 1*, (*FKF1*) (*LKP3/ADO3*), is an output component important for regulating flowering time, but does not affect the clock (Imaizumi et al. 2003).

The ZTL protein interacts with both PHYB and CRY1 in vitro (Jarillo et al. 2001), and loss-of-function *ZTL* phenotypes strongly implicate its involvement in the phototransduction pathway (Somers et al. 2000, 2004). Compared to the single mutants, both *ztl-phyB* and *ztl-cry2* double mutants under red light show enhanced period lengthening and shortening respectively, implying they act in parallel or additively in photoentrainment (Somers 2005).

12.2.2.3 The Role of ELF in Phototransduction

Early Flowering 3 (*ELF3*) encodes a PHYB-specific interacting protein that reaches peak expression towards evening, decaying to undetectable levels in constant dark (Covington et al. 2001; Hicks et al. 2001). Loss-of-function of *ELF3* results in arrhythmicity (McWatters et al. 2000; Covington et al. 2001; Hicks et al. 2001) whereas overexpression causes a light-dependent period lengthening, but under continuous darkness, neither mutation has any effect on period length (Covington et al. 2001). Interestingly, *elf3* mutations increase light sensitivity of photodependent processes, particularly at night, and *ELF3* overexpression diminishes phototransduction (Covington et al. 2001). Furthermore, the absence of *ELF3* appears to affect the clock only after 10 h in constant light and the normal "clock-gated" acute response to light is largely absent in *elf3* mutants (McWatters et al. 2000). Thus, ELF3 appears to modulate the activity of the light input pathways around dusk and into the night (McWatters et al. 2000; Covington et al. 2001). *ELF3* therefore functions as a zeitnehmer – a "time bringer" (Roenneberg and Merrow 2000).

Similarly, *ELF4* mutations result in arrhythmicity (Tepperman et al. 2001; Doyle et al. 2002), but in contrast to *elf3* mutants, *elf4* mutations disrupt rhythmicity in constant darkness too. *ELF4* expression is also under the clock control, with peak abundance corresponding to that of *ELF3*, indicating some association between the two proteins (Doyle et al. 2002).

Patterns in ZTL protein levels also show pronounced light/dark cycles, with peak levels corresponding with those of ELF3 (W.Y. Kim et al. 2003; Somers et al. 2004). Thus, a physical interaction between ELF3 and ZTL appears possible, although the effect on the circadian system is largely additive (Kim et al. 2005).

12.2.2.4 Time For Coffee

Time For Coffee (*tic*) mutants also result in loss of precision of circadian rhythms, with shortened free-running periods and disrupted circadian gating (Hall et al. 2003). Significantly, in contrast to ELF3, TIC arrests clock function in the morning (Hall et al. 2003). Thus, ELF3 is considered to gate the clock at night, with TIC having an analogous role during the day. This is supported by observations that *tic1-elf3* double mutants are fully arrhythmic under light/dark cycles and show no anticipation of dawn (Hall et al. 2003).

12.2.2.5 Sensitivity to Red Light

Originally identified as a mutant impaired in PHYB signalling, *SRR1* (*Sensitivity to Red light Reduced 1*) now appears to have a wide-ranging effect on circadian cycling. Under constant red light, all clock genes tested show a shorter period, more rapid dampening and reduced oscillation amplitude in the *ssr1* mutant (Staiger et al. 2003). Periods are similarly shortened under constant dark, indicating a role beyond only PHYB-mediated transduction. Furthermore, as SRR1 does not appear to interact directly with PHYB (Staiger et al. 2003), it seems that other components lie between the photoreceptor and SRR1.

12.2.2.6 Gigentea

The actual role of GI (Gigentea) is unresolved, although *gi* mutants suggest a connection with light input to the oscillator (Fowler et al. 1999; Park et al. 1999). GI may also be associated with PHYB function (Huq et al. 2000). Alternatively, GI could form a feedback loop in oscillator function because it is required for high-amplitude expression of *CCA1* and *LHY* (Fowler et al. 1999; Park et al. 1999; Mizoguchi et al. 2002). *GI* is clock regulated, thus is also a clock output (Park et al. 1999).

12.2.2.7 The APRR Circadian Wave

The four *Pseudo-Response Regulator* (*APRR*) genes, the APRR quintet (Matsushika et al. 2000; Strayer et al. 2000), are expressed rhythmically in sequence: *APRR9-APRR7-APRR5-APRR3-TOC1*, every 2 to 3 h from dawn to

dusk (Makino et al. 2002); the so-called "circadian wave" (Matsushika et al. 2000). All members have an (albeit variable) effect on the free-running period, as demonstrated by single-mutant studies and overexpressing lines (Eriksson et al. 2003; Farré et al. 2005). Importantly, some of the single-gene knockout mutants show no circadian defect in constant darkness, although short and long free-running periods occur in constant light, implying a role in photoentrainment as opposed to the oscillator itself (Eriksson et al. 2003; Farré et al. 2005). Further support of this role is that *APRR9* expression is both light- (Makino et al. 2002) and phytochrome-activated (Matsushika et al. 2000).

However, the original model of the wave *APRR* family gene expression, leading to *TOC1* induction is not currently favoured (Hanano and Davis 2005). Supporting this rejection is that *APRR9* and *APRR5* function in blue light input to the clock, rather than red, whereas *APRR7* functions in red light input, rather than blue (Eriksson et al. 2003; Farré et al. 2005). Also, double-mutant studies indicate that *APRR9* and *APRR5* function in distinct pathways (Eriksson et al. 2003), and both *APRR9* and *APRR5* have a strong phenotype under blue light, but in opposite directions (Eriksson et al. 2003; Farré et al. 2005).

12.2.3 Negative Regulation of Photoentrainment

A key regulator of the specific targeting of proteins for ubiquitination and proteasome-mediated degradation is COP1 (Constitutive Photomorphogenesis 1). This E3 ubiquitin protein ligase acts downstream of both phytochromes and cryptochromes (Ang and Deng 1994) and controls light signalling by regulating the degradation of several key transcription factors (Osterlund et al. 2000). The COP1 protein is localised to the nucleus in the dark where it associates with the nucleus-located COP9 signalosome (Schwechheimer and Deng 2001). In the light, COP1 moves out of the nucleus, and proteins involved in the positive regulation of photomorphogenesis, such as HY5 (Elongated Hypocotyl 5), are no longer degraded (Karniol et al. 1998). *HY5* and other COP1-regulated transcription factors then accumulate and bind to light-regulated promoter elements (Liscum et al. 2003). CRY1 has been identified as part of the mechanism involved in moving COP1 from the nucleus to the cytoplasm in the light (Osterlund and Deng 1998) and both CRY1 and CRY2 physically interact with COP1 in a light-dependent manner, repressing COP1 activity. Furthermore, COP1 interacts with PHYA and PHYB, leading to their ubiquitination (Seo et al. 2003).

12.2.4 Temperature Entrainment

In *Arabidopsis*, gene expression can also be entrained by temperature cycles (Michael and McClung 2002; Salomé et al. 2002; Salomé and McClung 2005a). Transcription of the clock genes *CCA1*, *LHY* and *TOC1* is entrained by

thermocycles showing the same phase as during light/dark cycles, although with less acute induction or repression at temperature steps. This suggests that temperature entrains the clock at the posttranscriptional level (Salomé and McClung 2005a).

However, temperature signalling to the oscillator is poorly defined. One group of genes proposed to be involved is the *APRR* quintet. It has been suggested that the failure of the *aprr7-aprr9* double mutant to maintain circadian rhythms after temperature entrainment indicates the involvement of APRR7 and APRR9 in temperature input to the oscillator (Salomé and McClung 2005a). Under thermal cycles, the absence of *aprr5-aprr7* enhances the dampening effect of the amplitude of mRNA cycling under light/dark cycles (Nakamichi et al. 2005), implicating also these genes in temperature entrainment.

12.3 The Central Oscillator

The underlying pacemaker of the circadian system is a cellular autonomous oscillator. Through a series of biochemical reactions, it generates a self-sustaining cycle of approximately 24 h and imposes this rhythmicity onto physiological, biochemical and molecular events: the "hands of the clock". A major breakthrough in identifying the components of the oscillator was the development of the *promoter::luciferase* system to monitor circadian rhythms (Millar et al. 1992). *Arabidopsis* plants carrying a firefly *luciferase* (*LUC*) gene fused to the circadian-regulated *Chlorophyll a/b-Binding Protein* (*CAB*) promoter exhibit robust rhythms in bioluminescence that can be monitored with a photon-counting video camera (Millar et al. 1992). This has led to our current understanding of the *Arabidopsis* circadian system.

12.3.1 The *CCA1/LHY-TOC1* Model for the *Arabidopsis* Central Oscillator

The three best-described genes in the *Arabidopsis* circadian oscillator are: *Timing Of CAB 1* (*TOC1*), *Late Elongated Hypocotyl* (*LHY*) and *Circadian Clock Associated 1* (*CCA1*). *LHY* and *CCA1* encode MYB-like transcription factors with partly redundant functions (Schaffer et al. 1998). The *TOC1* gene encodes a nuclear protein, with an atypical receiver domain, similar to those found in other response regulator proteins (Strayer et al. 2000).

The reciprocal regulation between TOC1 and LHY/CCA1 appears to define the basic framework for the clock mechanism in *Arabidopsis* (Fig. 12.4; Alabadí et al. 2001). TOC1 oscillates with peaks in mRNA and protein abundance near dusk, approximately 180° out of phase with CCA1 and LHY, which peak around dawn. These negative elements LHY and CCA1 bind to and inhibit transcription of the positive element; the *TOC1* promoter, leading to decay in *LHY* and *CCA1* transcript levels during the course of the day.

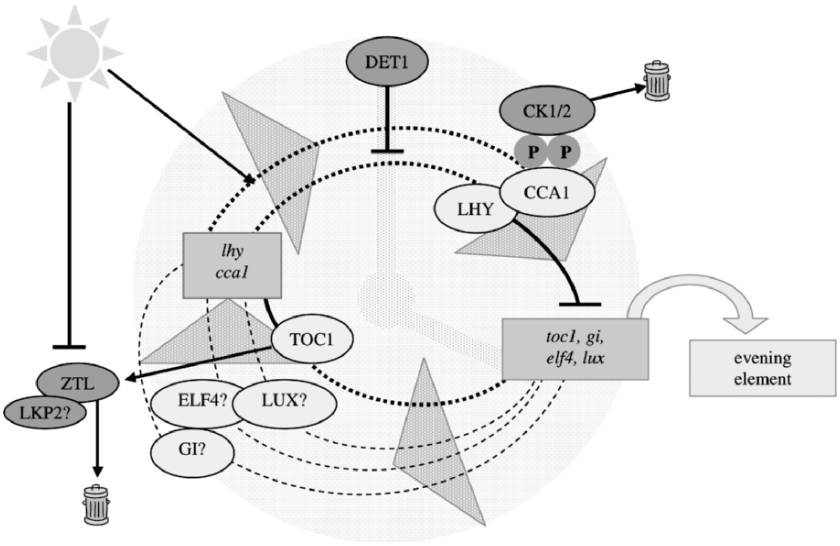

Fig. 12.4 The clock central oscillator: a model of the main components of the core clock. Genes are represented in *boxes* and proteins in *ovals. Bold lines* indicate transcription/translation (*dotted lines*) and protein activity (*solid lines*). *Question marks* denote putative lines in regulator connectors for proteins for which uncertainty remains

Decreasing levels of CCA1 and LHY derepress the *TOC1* gene, enabling its transcription to resume in the early evening. The subsequent accumulation of TOC1 protein at night activates transcription from the *LHY* and *CCA1* promoters through an unknown mechanism, thereby reinitiating the oscillatory cycle.

In support of their role in the central oscillator, constitutive overexpression of any of the components: *CCA1*, *LHY* or *TOC1*, abolishes circadian rhythmicity (Wang and Tobin 1998; Schaffer et al. 1998). Loss-of-function mutants of *LHY* and/or *CCA1* are incapable of sustained rhythmicity under constant conditions (Alabadí et al. 2002; Mizoguchi et al. 2002), and silencing of *TOC1* causes arrhythmia in darkness and constant red light (Más et al. 2003a).

However, the picture is incomplete. *lhy-cca1* double mutants for instance, retain a weak short-period rhythm under constant light (Alabadí et al. 2002; Mizoguchi et al. 2002), implicating additional components in the system.

Several other members of the APRR gene family to which *TOC1* belongs may also have a role in the core oscillator (Farré et al. 2005; Nakamichi et al. 2005). CCA1 regulates *APRR7* and *APRR9* expression by directly binding to a CBS (CCA1-binding site) in their promoters, and APRRs regulate *CCA1* and *LHY* expression (Farré et al. 2005). In addition, *aprr7-aprr9* double mutants have extremely long periods under constant conditions, consistent with a role in the core oscillator (Farré et al. 2005; Nakamichi et al. 2005). Nonetheless, the position of APRRs in the core oscillator remains ambiguous (Farré et al. 2005).

ELF4 is required for maintenance of rhythmic outputs and for light-induced expression of *CCA1* and *LHY*, but not *TOC1* and it is repressed by *CCA1* and *LHY* (Doyle et al. 2002; Kikis et al. 2005). As the expression profile of *ELF4* parallels that of TOC1, a negative feedback loop formed by *CCA1/LHY* and *ELF4* has been proposed (Kikis et al. 2005).

GI expression and protein levels peak in the late subjective afternoon, again paralleling that of *TOC1* (Fowler et al. 1999). Helping to maintain robust rhythms, GI also feeds back to the clock, implying a central oscillator role (Fowler et al. 1999; Park et al. 1999).

Mutation of the *LUX* (*lux Arrthmo*) (Latin for light)/*PCL1* (*phytoclock*) gene leads to arrhythmia under constant conditions, with *CCA1/LHY* and *TOC1* clamped high (Hazen et al. 2005). Overexpression of *LUX* reduces the levels of expression of both *GI* and *LUX*, and dampens oscillations of *LUX* as well as *TOC1*, *CCA1* and *LHY* in continuous light (Onai and Ishiura 2005). Similarly to CCA1 and LHY, the predicted LUX protein contains a single MYB DNA-binding domain. It is circadian regulated, expressed coordinately with *TOC1* and is negatively regulated by the same set of proteins that contribute to *TOC1* expression (Hazen et al. 2005), suggesting that LUX may participate in the clock in a manner analogous to that of TOC1.

12.3.2 Is There more than One Oscillator Within Plants?

In contrast to mammals where a core oscillator is located in specialised cells in the suprachiasmatic nucleus of the brain and semi-autonomous "slave" oscillators occur in the peripheral tissues (Roenneberg and Merrow 2000), plants lack an obvious central pacemaker (McWatters et al. 2001). Thus, the organisation of the plant circadian system is likely to stand apart from the hierarchical system in animals. Evidence suggests that timekeeping in plants relies on independent, multiple clocks directing a plethora of rhythmic outputs (Millar 1998; Webb 1998).

Individual organs and even every cell in plants may have an independent oscillator, entrained independently of oscillators in neighbouring cells. Numerous studies show desynchronisation in the periods of rhythmic outputs between distinct tissue types. Light entrainment in one organ does not affect entrainment in the rest of the plant, suggesting that the clocks are autonomous, lacking extensive crosstalk, even within the same tissue. For example, in *Arabidopsis*, *CAB2::LUC* luminescence persists in cultured cells (Sai and Johnson 1999) and in isolated explants, and *CAB2::LUC* luminescence rhythms can be entrained to opposite phases in the same leaf (Thain et al. 2002). These oppositely phased rhythms are maintained in constant light, indicating that the oscillators driving the rhythms remain uncoupled (Thain et al. 2002). The periods of leaf movement and *CAB2::LUC* luminescence rhythms also sometimes differ markedly (Millar et al. 1995), as can the periods of leaf movement and stomatal conductance (Hennessy and Field

1992). Such a high degree of autonomy is consistent with the absence of a central pacemaker.

The most compelling evidence for the existence of oscillators with different properties comes from studies of temperature and light entrainment. *CAB2* is entrained preferentially by light/dark cycles, whereas *CAT3* (*Catalase 3*) is set by temperature cycles and is insensitive to photoperiod (Michael et al. 2003). The luminescence rhythms in the *CAB2::LUC* and *CAT3::LUC* lines have distinct phase responses to low-temperature pulses. Since the two promoters drive *LUC* expression in similar tissues, there is evidence of the presence of two circadian oscillators, possibly within the same cell, with different sensitivities to entraining signals. However, to determine conclusively the presence of multiple oscillators within cells requires simultaneous measurement of both promoter activities over a circadian time course within a single cell.

An alternative hypothesis is that there may be no core molecular oscillator, but rather multiple independent feedback loops that contribute collectively to time-keeping (Roenneberg and Merrow 2000; Lillo et al. 2001). However, if multiple oscillator types are present in different cells or tissues, then it is likely that they have a broadly similar molecular architecture. Indeed, mutations in the major components of the clock *LHY/CCA1* and *TOC1* shorten all circadian outputs tested to date (Millar et al. 1995; Somers et al. 1998b; Strayer et al. 2000).

12.3.3 Regulation of the Circadian Oscillator

The circadian oscillator genes have rhythms in transcript abundance, followed by rhythms in the abundance of the cognate proteins (Dunlap 1999). These proteins bind to promoter elements in other clock genes and initiate their expression. In turn, the secondary proteins bind to the promoter elements in the initial gene and negatively regulate its expression. This process generates rhythms in the steady-state transcript and protein abundance of both components (Roenneberg and Merrow 2000; Alabadí et al. 2001). However, at least one component must show a significant delay between the expression of the transcript and the expression of the active protein to maintain the 24-h period and prevent attenuation of the rhythms (Dunlap 1999). Control of protein degradation is integral to the maintenance of robust circadian oscillation, contributing to these necessary delays.

The proteins phosphorylated by CK1, CK2 and other kinases are targeted for degradation via the ubiquitin-proteasome pathway (Más et al. 2003b). This involves the addition of a poly-ubiquitin chain to the protein, a process mediated by the SCF (Skp1-F-box-Cullin; del Pozo and Estelle 2000). In *Arabidopsis*, the protein kinase CK2 phosphorylates CCA1 in vitro (Sugano et al. 1998) and CCA1 phosphorylation by CK2 appears to be necessary for the normal functioning of the *Arabidopsis* clock in vivo (Daniel et al. 2004). Such processes may be involved in the regulation of the core components of the

central oscillator, and a number of proteins have been identified that may be important in the ubiquitin-proteasome pathway.

ZTL targets the degradation of TOC1, thereby modulating the circadian period (Somers et al. 2000; Más et al. 2003b). Supporting this is that TOC1 is more stable in a *ztl* background, graded increases in ZTL expression cause a corresponding shortening in free-running period and TOC1 and ZTL physically interact in planta (Han et al. 2004).

Phenotypes similar to *ZTL* are seen in plants overexpressing *LKP2*, a closely related gene (Jarillo et al. 2001; Schultz et al. 2001), suggesting that *LKP2* may also lie close to the clock. Mutant studies indicate redundancy with *ZTL* and it is likely to act in a similar manner (Schultz et al. 2001; Somers et al. 2004; Salomé and McClung 2005a, b).

DET1 (De-Etiolated 1) promotes ubiquitination and proteolytic degradation of specific target proteins (Yanagawa et al. 2004), in a manner analogous to that of COP1. DET1 appears to function in a light-independent pathway modulating *LHY* expression at the posttranscriptional rather than the transcriptional level (Song and Carré 2005).

Other posttranslational mechanisms that affect the functioning of the circadian clock include rhythmic activation by phosphorylation (Iwasaki et al. 2002), modification of protein-protein interactions, alteration of subcellular locations (Harms et al. 2004; Meyer et al. 2006), and poly(ADP-ribosyl)ation as indicated by the *tej* mutant (Panda et al. 2002).

12.4 Outputs of the Circadian System

Arabidopsis exhibits a myriad of rhythmic outputs or "hands of the clock", and the list of clock-controlled genes (CCGs) has expanded considerably in recent years. Microarray and enhancer trapping experiments suggest that up to 36% of the *Arabidopsis* genome is under circadian regulation (Harmer et al. 2000; Schaffer et al. 2001; Michael and McClung 2003). These transcripts show peak abundance at all phases of the day and night, evidence that a complex network of transcription factors is used to generate this high variety of phases.

A large proportion, estimated at 68%, of the rhythmically regulated genes also directly respond to environmental stress (Kreps et al. 2002). Rhythmic expression of these genes in anticipation of predictable environmental changes might prepare the plant to withstand an expected stress, or make best use of a resource, so that circadian regulation would complement the plant's subsequent response to the stress (Green et al. 2002). Photoperiodism is a special case of clock control in which a circadian rhythm is combined with light signalling. The photoperiod sensor enables plants to respond to the annual cycle of day-length, making flowers, tubers or frost-tolerant buds at the appropriate season, and the selective advantage of correct seasonality are clear (Hayama and Coupland 2003).

An important question raised by the identification of the large number of CCGs is how their differing circadian patterns of expression are generated. Microarray experiments, combined with computational analysis, have helped to identify common motifs in the upstream regions of co-regulated genes. A nine base-pair sequence termed the "*Evening Element*" (*EE*) is overrepresented in a cluster of genes of which the expression peaks at the end of the day (Harmer and Kay 2005), suggesting that this motif plays an important role in the regulation of evening-phased genes. Furthermore, the *EE* is implicated in the circadian regulation of *TOC1*, *GI* and *ELF4*, implying that it may play a role in the functioning of the central oscillator itself (Harmer et al. 2000; Alabadí et al. 2001). A related motif, the *CCA1-Binding Site* (*CBS*), may impart dawn-phased rhythms, mediated by the positive action of CCA1 and LHY (Michael and McClung 2002). However, these may not act alone to specify circadian phase – another closely related motif, the "*Morning Element*" (*ME*) has recently been identified (Harmer and Kay 2005).

Another motif observed more frequently in the promoters of CCGs than expected is the G-box found in the promoters of many light-regulated genes (Hudson and Quail 2003; Michael and McClung 2003). However, the functional significance of this has not yet been reported.

12.5 Concluding Remarks

The progress achieved in the last few years towards unravelling the plant circadian clock mechanism is remarkable, but much remains unfinished. Although we can safely conclude that the paradigm of interlocked feedback loops constituting a circadian oscillator is confirmed in plants, not all the components have been identified. Details of entrainment of the clock and our knowledge of how the outputs are generated are severely lacking. Indeed, the mechanistic details of almost every step are incompletely understood. However, if the recent progress made continues, we should eventually fully understand how the endogenous circadian clock works.

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Part 4
Theoretical Aspects of Rhythmical Plant Behaviour

13 Rhythms, Clocks and Deterministic Chaos in Unicellular Organisms

David Lloyd

Abstract

The cell generation or cell cycle time in a clonal population of identical unicellular organisms growing under steady-state conditions in a carbon-(or energy-) limited continuous culture (chemostat) shows a broad distribution. This indicates that the rate of cell division cycle traverse shows considerable variability. The basis for this variable temporal organization has been the subject of a great deal of speculation and many models have been suggested. This process is highly temperature-dependent and, similarly to all chemical and biochemical reactions, its rate approximately doubles for every 10 °C rise over a certain range of growth temperatures (i.e. the $Q_{10} \simeq 2$). Clock-controlled biological processes, on the other hand, are temperature-compensated, so that $Q_{10} \simeq 1$. Two well-established examples are the circadian ($\tau \simeq 24$ h) and the ultradian clocks ($\tau \simeq 0$ min in *Saccharomyces cerevisiae*). Other biological processes proceeding in faster time domains often show oscillatory dynamics. A well-studied example is glycolysis, although a function for glycolytic oscillations is not yet established. As all these examples depend on three or more variables, and are often in coupled sets, departure from regular oscillatory behaviour into deterministic aperiodicity is to be expected. Chaos has been demonstrated in biochemical (enzyme-catalysed) as well as chemical reactions. Furthermore, it has been shown in a metabolic pathway (glycolysis) and in the complex system of the cell division cycle. Chaos may also arise from coupled oscillators. One possible mechanism for spatiotemporal coherence (order) arises in unicells as an output from a tuneable multi-oscillator (controlled chaos). Biological functions for these oscillatory phenomena are discussed.

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S. Mancuso and S. Shabala (Eds.)
Rhythms in Plants: Phenomenology, Mechanisms, and Adaptive Significance

13.1 Time in Biology

The integrated and coherent behaviour of living systems remains as the central unsolved problem of biology (Lloyd and Gilbert 1998). The most recent attempts to achieve a synthesis bring several powerful new techniques to bear on this age-old enigma (Lloyd 2005). In a universe where increasing disorder rules, driven by ineluctable thermodynamic forces, islands of self-organization may seem only remotely possible (Wicken 1987; Schneider and Sagan 2005). Yet, provided that conditions are held within a set of defined boundaries, the development and maintenance of the living state proceed apace and in a truly robust manner. Explanations require more than biochemical understanding; in addition to details of the molecular structures of components, their synthesis, interconversion and control (and the kinetics of these processes), we need to be able to probe integrated physiological functions at both descriptive and mechanistic levels (Weber 1990). For higher organisms, the latter has a long and distinguished history, albeit at the level of the isolated organ, rather than as an orgasmic entirety (Boyd and Noble 1993; Noble 2002).

Successive attempts to come to terms with the problem at a theoretical level have included systems theory (von Bertalanffy 1952, 1968), cybernetics (Wiener 1961) and, today, "systems biology" (Lloyd 2005; the latter is actually not a new concept but one made ever more feasible by new techniques). Matching the ideas emanating from control engineering with biochemical information on metabolic control has yielded exciting and valuable results (Kacser and Burns 1973; Heinrich and Rapoport 1974) and insights (Fell 1997, 2005; Cortassa et al. 2002). Even before the power of digital computing was unleashed upon theoretical biology, realistic simulations of glycolytic control were obtained using analogue methods (Garfinkel 1971). The discovery of metabolic oscillations (Chance et al. 1964, 1973) gave impetus to these activities (Higgins 1963).

Timescales (Fig. 13.1) within which biological oscillations operate span many decades of seconds (Lloyd 1998); in plants, oscillations in photosynthetic events may have periods of femtoseconds; attosecond events underlie molecular interactions. Circadian rhythms dominate biological time. In plants, however, infradian processes (seasonal changes and annual rhythms) are often even more obvious. On ecological and evolutionary timescales, oscillatory behaviour is also well documented.

Thus, life is characterized by spatiotemporal complexity on spatial scales that range from the infinitesimal to the global, and over 30 decades of time measured in seconds (Aon and Cortassa 1997). Methods for studies on these different timescales are to a large extent now available (Lloyd et al. 1982b), although the time spans corresponding to our "spectrum of ignorance" (Kamen 1963) have not been completely elucidated.

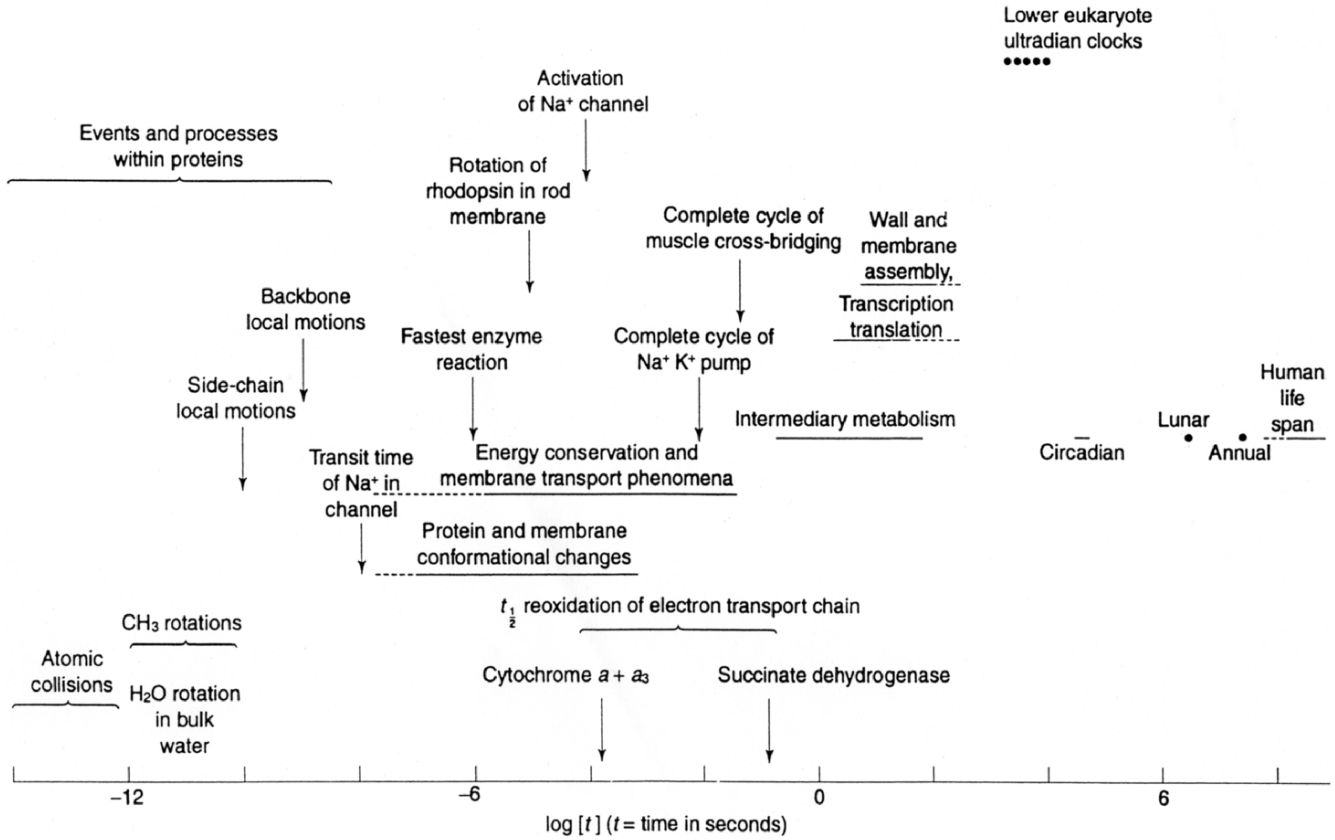

Fig. 13.1 The time domains of living systems. Approximate relaxation times (to a return of 1/e following perturbation) are plotted against time in seconds (log scale)

13.2 Circadian Rhythms

The earth has turned a million million times since the dawn of life. For all this time, evolution in a periodic environment has led to the development of organisms finely tuned to exploit all the possible advantages of rhythmic performance (Bünning 1964; Edmunds 1988; Lloyd and Rossi 1992; Dunlap et al. 1999; Chandrashekaran 2005). Thus, one of the most characteristic properties of living organisms is their inherent rhythmicity – their autodynamic nature (Lloyd and Gilbert 1998). As objects of the highest complexity, their constituent functions operate over a wide range of timescales (Fig. 13.1) and, on each of these timescales, coordination, regulation and control is paramount. The system has to be coordinated with the external environment. The most evident changes to which organisms must accommodate and that they must anticipate are those necessitated by the cycles of day and night. Thus, the most evident periodicity in performance is a 24-h cycle, the circadian cycle. Strong survival pressures have ensured the ubiquity of an endogenous clock, reset twice daily by dawn and by dusk, and also providing an anticipatory advantage. Thus, when reference is made to "the biological clock", it is the daily circadian clock that is being alluded to.

13.2.1 Circadian Timekeeping in Unicellular Organisms

Circadian clocks occur in all organisms from cyanobacteria to humans; their presence in some other bacteria has been claimed but is still a matter of dispute (Halberg et al. 2005). Several examples of eukaryotic unicellular organisms (*Chlamydomonas*, *Tetrahymena*, *Paramecium*, *Gonyaulax* now renamed *Lingulodinium*, and yeasts) have provided excellent systems for study, as has also the mycelial fungus, *Neurospora*. Many of the fundamental advances in understanding have come from studies of these organisms and have resulted in an enormous literature, reviewed earlier by Lloyd (1998) and more recently by Lakin-Thomas (2000), Lakin-Thomas and Brody (2004) and Gillette and Sejnowski (2005). Functional proteomics may enable discovery of novel components of the circadian system (Wagner et al. 2005).

13.2.2 Cyanobacterial Circadian Rhythms

The prokaryotic *Synechococcus elongatus* has three clock genes *KaiA*, *KaiB* and *KaiC*, with the latter two genes as a coordinately transcribed operon. A negative feedback autoregulatory loop operates in this system. As many as 9% of the genes in this cyanobacterium exhibit circadian cycling (Kucho et al. 2005), with functions that include a diverse spread of activities (some metabolic pathways, membrane transport, respiration, poly3-hydroxyalkanoate synthesis, and signal transduction, as well as genes involved in transcription

and translation). The central mechanism of the cyanobacterial system is quite distinctive from that of all eukaryotic systems studied, and this unique system has been reconstructed in vitro (Nakajima et al. 2005) using purified clock proteins (KaiA, KaiB and KaiC) in the presence of ATP. The phosphorylation state of *KaiC* oscillates with a ca. 24-h period. The clock assembles and disassembles with the passing of each day and may be regarded as a special organelle, the "periodosome" (Kageyama et al. 2003). Individual bacteria show strong temporal stability of their robust clock output and it appears, therefore, to be a property of the intracellular biochemical network (Mihalcescu et al. 2004), with no requirements for cell–cell communication.

The oscillator shows temperature compensation and is undamped. When mutant clock proteins were used to construct the in vitro system, its period reflected that seen in vivo. An hourglass model for this system highlights the importance of collective assembly–disassembly events in biochemical networks (Emberly and Wingreen 2006). The light-driven entrainment of the cyanobacterial clock is mediated by protein encoded by *LdpA* (Ivleva et al. 2005). This protein contains two [4Fe4S] clusters and is sensitive to the redox state of the organism.

The absence of the *Kai* genes in the genome of green algae and in higher plants is rather surprising, as the chloroplast is widely considered to be derived from an ancestral cyanobacterium.

13.3 Ultradian Rhythms: the 40-Min Clock in Yeast

As well as becoming entrained and matched to the daily external environmental changes, there is another timekeeping requirement for life. This is the internal time base, upon which all the diverse biochemical events, sequences and processes must be coordinated. Timescales in this case are much less than a day; hence, the high-frequency processes involved may be called "ultradian" (Lloyd and Stupfel 1991) or, more precisely, "circahoralian" (Brodsky 1975, 2006). Time domains here cover many decades when measured in seconds on a logarithmic scale (Fig. 13.1) – from attoseconds (eg. trapping of photons in the photosynthetic light absorption events), through picoseconds (eg. ligation of CO to the haem of haemoglobin), nanoseconds (eg. fluorescence energy transfer phenomena), microseconds (eg. conformational state changes in membranes), milliseconds (eg. electron transport steps), seconds (eg. metabolic interconversions) and minutes (eg. transcription) to hours (eg. cell divisions). On and between all these levels of temporal hierarchy, there is a necessity for timekeeping, so that convergence of parallel processing is ensured and so that the whole system is coherent (Lloyd and Murray 2005). The analogy with a computer is apt (albeit partial). The magnificent, miraculous machine also creates itself as it performs, and creation requires continuing destruction – complexity par excellence!

The most detailed elucidation of an ultradian clock comes from the yeast, *Saccharomyces cerevisiae*, in which a 40-min periodicity has been revealed. It is stressed that this phenomenon is neither a special case nor a mere curiosity. The strict rhythmic self-organization evident here is required in every living organism; its pervasive ubiquity underlies its importance. In other yeasts, its period is different, as is the case in protozoans, (Table 13.1), in plants and in humans; the time base that the ultradian clock provides is a necessity for all life processes.

Table 13.1 Ultradian clock-driven outputs in lower eukaryotes

Organism	Period (min)	Measured variable
Yeasts		
Saccharomyces cerevisiae	42	Respiration [O_2, CO_2] Fermentation [ethanol, acetaldehyde, acetic acid] H_2S evolution Redox state [NADPH, GSH] Mitochondrial structure, inner membrane potential Transcription Replication of DNA
Schizosaccharomyces pombe	40	Respiration [O_2, CO_2] Adenine nucleotides [ATP, ADP, AMP] Mitochondrial ATPase NADH and flavin redox state Septum formation
Candida utilis	33	Respiration [O_2]
Protozoa		
Acanthamoeba castellanii	69	Respiration [O_2] Adenine nucleotides [ATP, ADP, AMP] Total protein, RNA Flavin fluorescence Enzyme activities Cytochromes
Crithidia fasciculata	66	Respiration [O_2] Adenine nucleotides [ATP, ADP, AMP]
Paramecium tetraurelia	69	Motility Cell division
Tetrahymena pyriformis	50	Respiration [O_2]
Dictyostelium discoideum	60	Respiration [O_2] RNA, total protein
Algae		
Chlamydomonas reinhardii	55	Chlorophyll a
Euglena gracilis	240	Tyrosine aminotransferase

The yeast system has many advantages; its most important property is the availability of continuous readout methods, so that the clock output may be followed second upon second, minute upon minute, hour upon hour, day upon day, up to several months. In a continuous culture vessel, temperature, pH, stirring rate, aeration rate and nutrient supply are strictly and automatically controlled (Monod 1950). Residual dissolved O_2 is monitored continuously and, as the supply rate does not vary, any changes in O_2 consumption are reflected in a reciprocal change in dissolved O_2 levels, i.e. we are measuring the respiratory activity of the organisms (Harrison 1973; Pirt 1975). Initially, cells are grown in a batch culture in which, near the onset of nutrient and stored-energy reserve depletion, the dissolved O_2 begins to oscillate (Murray 2004). Continuous growth is then initiated after all the carbon sources have been exhausted, by starting the culture medium flow. The immersed O_2 electrode provides a stable device for continuous measurement. The output is not sinusoidal but rather has a distinct waveform; its trough-to-peak amplitude is about half that of the highest levels of dissolved O_2 attained, and its period about 40 min (Fig. 13.2; Satroutdinov et al. 1992). Experimental changes of temperature through a series of discrete steps reveal the clock-like characteristic of the compensation of period length (Murray et al. 2001). Other characteristics suggest that well-known signalling pathways are involved; these include dose-dependent prolongation of period length by Li^+, and perturbation of waveform and period by A-type monoamine oxidase inhibitors such as phenelzine (Salgado et al. 2002).

One of the most intriguing questions posed by this oscillating yeast culture is the basis of its coherent behaviour. If each organism were performing all its legion tasks independently, then we would see a steady state in which time averaging across the population would result in a time-independent output. In fact, the individuals (more that 10^9 of them) have become synchronized and, as the oscillations of dissolved O_2 are of such large amplitude, this synchrony must have involved a majority of individuals. Essential to the respiratory synchrony exhibited is the pulsatile release of H_2S (a potent inhibitor of cytochrome *c* oxidase, the terminal component of the mitochondrial respiratory chain). This volatile small molecule is rapidly oxidized by O_2 dissolved in the culture, and so shows a series of spikes at 40-min intervals (Sohn et al. 2000; Sohn and Kuriyama 2001). Another transiently produced and rapidly eliminated volatile messenger substance, responsible for extracellular communication between cells of the culture, is acetaldehyde, a fermentation product of the yeast (Murray et al. 2003). The importance of both these compounds and possibly that of other related, easily diffusible chemical species has been systematically shown by their phase-shifting capabilities when added to culture at certain times during the 40-min cycles.

Deletion of a single yeast gene *GTS1* affects the ultradian clock. Whereas it has been claimed (Mitsui et al. 1994) that complete loss of rhythmicity is a consequence, a more recent study (Adams et al. 2003) indicates a change of period

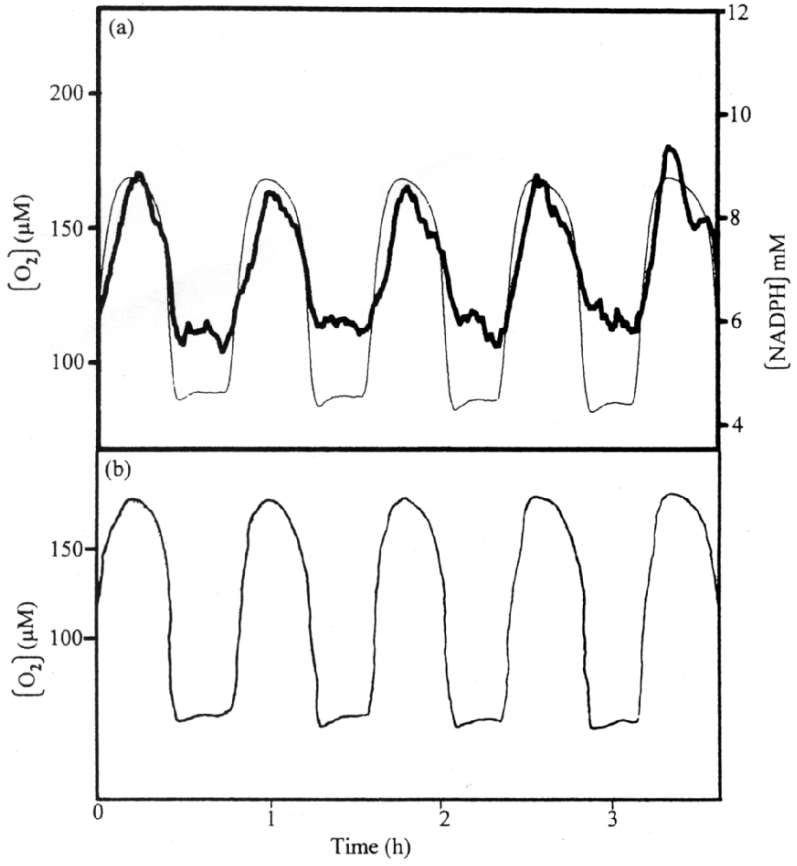

Fig. 13.2 Dissolved O_2 and reduced nicotinamide nucleotide fluorescence in a continuous culture of *S. cerevisiae*. **a** Simultaneous O_2 electrode and fluorimetric monitoring (366→450 nm) in the same culture: fluorescence emission was calibrated by nicotinamide nucleotide extraction from the culture. **b** Control culture (no UV irradiation)

of the respiratory oscillations from 40 to 18 min, occasional loss of stability, and a partial loss of temperature compensation. Thus, whereas the wild-type yeast has an ultradian clock period with a Q_{10} of 1.07, that of *GTS1* is 1.6. The waveform of the oscillation was also affected; the phase of enhanced respiration was more rapid, and there was a slower increase to the respiratory maximum. The mean concentration of acetaldehyde produced by the mutant (1.6 mM) was higher than that by the wild type (1.0 mM) but the peak–trough amplitude (1.0 mM) was similar in both. H_2S production rate showed a decreased amplitude in the mutant (1.55 and 0.80 $\mu mol/g_{cell}/h$ respectively).

As well as dissolved O_2, other redox active components show 40-min cycles. Although its waveform is more complex, NAD(P)H also shows a clear phase relationship with that of dissolved O_2, as does GSH (Murray et al.

1999). Therefore, the observed respiratory cycles are characterized by redox switching. Further evidence for this comes from the perturbative effects of 5-nitro-2-furaldehyde (a GSH synthesis inhibitor) and DL-butathionine (*S*,*R*)-sulphoximine (a γ-glutamyl cysteine inhibitor). Moreover, additions of either GSH or GSSG perturbed the respiratory oscillation. Alterations of phase and amplitude can also be produced by various agents that generate nitrosonium cations (NO^+), e.g. sodium nitroprusside, $NaNO_2$ or *S*-nitrosoglutathione; thiols and metalloproteins are the most likely targets for these agents (Murray et al. 1998).

Mitochondria undergo energization to produce the accelerated respiratory rates observed in continuous cultures when the oscillations of dissolved O_2 are at their minimal phases. Thus, during the 40-min cycle, the primary changes involved correspond to those described in isolated mitochondria, when they already have excess respiratory substrates but become fully activated only when supplemented with additional ADP. That massive changes in ionic balances across the inner mitochondrial membrane accompany this transition (and its reversal to restore the initial state) occurring on completion of ADP phosphorylation is well documented. Mitochondrial events that accompany respiratory oscillations include changes in cytochrome redox states, in inner mitochondrial membrane potential (as measured by fluorimetric assay using Rhodamine 123, a cationic dye internalized by mitochondria) and in conformational states (as revealed in electron micrographs of sectioned fixed yeasts sampled at peak and trough dissolved O_2 levels; Lloyd et al. 2002a, b). Confirmation that energy conservation within mitochondria is closely linked to the changing rates of respiration responsible for the respiratory oscillation came from studies on the effects of addition of uncouplers of mitochondrial energy-yielding reactions (protonophores; Lloyd 2003). Addition of 5 μM S_{13} produced a succession of changes. At first (within minutes), respiration of the yeasts shows the expected acceleration; this was most marked when protonophore addition occurred when the O_2 consumption was less than maximal in the respiratory cycle. Thus, the immediate effect was to attenuate the amplitude of the oscillation to an extent where it was no longer evident. Recovery occurred as the protonophore became diluted by the flowing medium and, after about 10 h, the respiratory oscillation had almost resumed its full amplitude. However, a further effect was observed; subsequent 40-min cycles were modulated by a slower rhythm with an 8-h cycle time. At the medium dilution rate employed (0.085 per hour), this interval approximated to the cycle time of budding. Similar events were observed with CCCP as with the protonophore S_{13}. When a culture that has spontaneously become synchronized to the cell division cycle was perturbed using 5 μM CCCP, the effect was to prolong the cycle time by 8 h. These data strongly suggest that the ultradian clock controls mitochondrial redox switching, phosphorylation capacity via energization state, and cell division cycle progression. The dynamics of responses clearly indicates that control is not dominated by mitochondrial events, as the kinetic competence of the organelle (e.g. as manifest in isolated organelles) is far

greater than that of the observed changes (Lloyd and Edwards 1984). The slow dynamics is imposed by other requirements in vivo, presumably by transcriptional switching from biosynthetic to degradative modes.

Analysis of genome-wide transcriptional activity during respiratory oscillations (Klevecz et al. 2004) showed that, of the 5,329 expressed transcripts, only 161 gave trough–peak differences greater than threefold. However, the transcripts could be assigned to two blocks or redox superclusters – those 650 that gave maximal expression during the active respiration phase or the oxidative phase of the cycle, and the 4,679 that were maximally observed during the "reductive" phase of the cycle. Furthermore, a subpopulation of organisms (5–10%) is gated in the transition between the G_1 and S-phase every oscillation cycle, during the onset of the low-respiration phase. Therefore, most of the DNA synthesis and RNA synthesis occurs when the cells are in a reductive state.

This temporal compartmentation may represent an evolutionary strategy to avoid oxidative damage to DNA and RNA. It is conceivable that originally, some 1.8 billion years ago, a sulphur-reducing proto-eukaryote could have employed this device to form a symbiotic relationship with the sulphide-oxidizing proto-mitochondrion using such a timekeeper, and that the separation of oxidative and reductive states are so necessary that present-day organisms have retained this central redox switch mechanism (Searcy 2003).

The global outputs of the oscillation imply a more complex mechanism than can be explained by a simple limit cycle created from a local, small-scale protein network. The robustness of the oscillator to changes in the external environment (pH, temperature, carbon source) supports a more complex global mechanism; a simple feedback oscillator would be intrinsically fragile to perturbation. Rather, it seems likely that the oscillation mechanism involves a massive array of weakly coupled oscillators. The array would be comprised of the majority of cellular processes.

In conclusion, the oscillation that characterizes the ultradian clock (Fig. 13.3) percolates not only throughout the population but also within the entire cellular network, including organelles, transcriptome, metabolome and proteome. This oscillatory state is not an exceptional curiosity found only in a peculiar system but rather a universal trait, necessary for the maintenance of the robust metabolic autodynamic state characteristic of normally healthy cells. Further consequences include the quantization of cell division cycle times (cell doubling times) as integral values of ultradian clock cycle times. We have determined ultradian clock cycle times for 10 different strains of *S. cerevisiae* as well as for 10 other yeast and protist species (Table 13.1; Lloyd 1992). The human basic rest activity cycle (ultradian clock) has a cycle time of 90 min (Anserinsky and Kleitman 1953).

Aging of cells is widely held to be due, at least in part, to an accumulation of reactive oxygen species-produced damage to cellular components, especially lipids and proteins. As oxidative stress varies during the mitochondrial energization cycles, this leads to the intriguing hypothesis that cell aging can

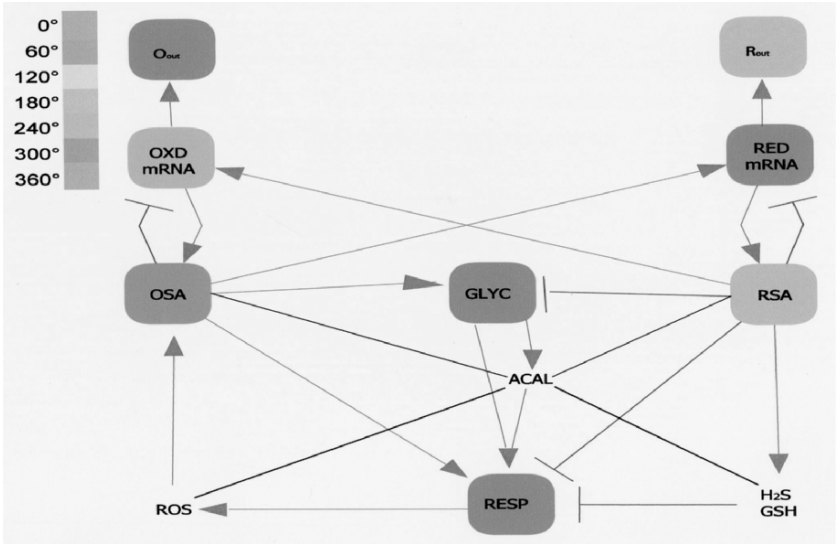

Fig. 13.3 The ultradian clock (composed of a massive array of metabolic and transcriptional elements) self-optimizes to produce an output characteristic of the biological species in which it is studied. Its core is a redox sensing network (for details, see Lloyd and Murray 2006) and, cycling between oxidative and reductive states, it partitions the ultradian clock so as to mimic a basic rest–activity cycle. It is the evolutionary precursor of circadian rhythmicity, and today is still responsible for circadian organization and that on longer timescales (seasonal, annual)

be quantitatively measured in ultradian cycles (Lloyd et al. 2003). Similar ideas have been expressed for circadian rhythms (Hardeland et al. 2003).

13.4 Oscillatory Behaviour During the Cell Division Cycles of Lower Organisms

Earlier work has been reviewed by Lloyd et al. (1982b) and Edmunds (1984, 1988). Oscillations in continuous cultures of yeast are commonplace (Sheppard and Dawson 1999). These open systems, especially when of large volume (e.g. on pilot plant or full industrial scales) and even under the most strictly controlled conditions, operate in pseudo-steady states, as reported in the vast literature on this topic. Many examples are typified by reactions to triggering events (transient anaerobiosis, pulsatile pH control by acid or alkaline titration, drop-wise nutrient addition, etc.). In other cases, synchronized cell growth and division (budding), as characterized by oscillatory O_2 consumption and partly as a consequence of changing trehalose utilization (Kuenzi and Fiechter 1969), leads to oscillatory states (Strässle et al. 1988;

Parulekar et al. 1986; Chen and McDonald 1990; Münch et al. 1992; Duboc et al. 1996). In these studies, dissolved O_2 and CO_2, proton production, and culture fluorescence were monitored online. Auberson et al. (1993) also used microcalorimetric measurements of heat evolution. Porro et al. (1988) showed the dependence of oscillations in respiration on dissolved O_2 levels. Cell size control is closely correlated with oscillations (Martegani et al. 1990); pH and carbon source also affect their properties (Beuse et al. 1999).

It should be emphasized that the overall cell cycle as well as its component processes are highly temperature-dependent and, hence, the frequently employed term "cell cycle clock" is, in fact, a misnomer. However, it has been treated as an oscillator-driven process ever since the pioneering concepts of Sel'kov (1970) and of Gilbert (1974), and many theoreticians have suggested candidates for the molecular components of the cell division control machinery and the mechanisms that drive cell division cycles (Norel and Agur 1991). Now that molecular genetics has provided many details on this topic, key control points have been revealed and many recently discovered oscillators described. Modelling proceeds apace (Mackey 1985; Mackey et al. 1986; Grasman 1990; Battogtokh et al. 2002; Tyson et al. 2003; Battogtokh and Tyson 2004a, b, 2006) and, provided that a full and realistic consideration of physiological scenarios is obeyed, new understanding of the variables important for biological phenomena (duration of cell cycle stages, identity of checkpoints) may emerge.

Gating of the cell cycle by the circadian clock has been studied extensively and, in the natural environment, this phenomenon is extremely important and widespread, from planktonic dinoflagellates (Sweeney 1982), cyanobacteria, the green alga *Chlamydomonas reinhardtii* and other protists (Mittag and Wagner 2003; Mittag et al. 2005) to the proliferative cells of humans in vivo and in culture (Klevecz 1992; Hammond et al. 2000; Schibler 2005). A mechanism involving cyclic AMP signalling has been established in *C. reinhardtii* (Carré and Edmunds 1993). Nuclear regulation of the chloroplast circadian expression has been confirmed (Matsuo et al. 2006).

13.5 Ultradian Gating of the Cell Division Cycle

13.5.1 Experimental Systems

We have observed in some protozoans (Lloyd et al. 1982a, b; Lloyd and Kippert 1987), as others did before us for cultured mammalian cells (Klevecz 1976), that cell division times are quantized. Using an established mathematical model of the cell cycle (Mustafin and Volkov 1977; Chernavskii et al. 1977), it is possible to see how quantization arises, provided that the ultradian clock modulates the mitotic oscillator (Lloyd and Volkov 1990, 1991). Interestingly, chaotic solutions of the model can be obtained over quite a wide range of parameter values. Furthermore, this model of Lloyd et al.

(1992) can explain the dispersion of cell cycle times through a population in strictly deterministic terms, whereas previous models involved stochastic effects (Brooks 1985) that necessitated provision of a "noise" term in the equations.

As an example of interacting oscillatory systems, we may take one that accommodates timekeeping for cell division. Here, recent experiments with yeast provide experimental validation of these theoretical predictions.

13.5.2 The Model

The following system of coupled differential equations (Chernavskii et al. 1977; Lloyd and Volkov 1990, 1991) was employed, in which the dynamics of the cell cycle oscillator depends on the concentrations of two constituents. Constituent L has a slow variable (τ_L, of the order of hours) and a fast variable (τ_R, of the order of minutes):

$$\begin{aligned} \tau_L \frac{dL}{dt} &= \eta - 2LR - DL \\ \tau_R \frac{dR}{dt} &= K + LR - R^2 - \frac{\gamma R}{(R+\delta)} \end{aligned} \tag{13.1}$$

where L and R are concentration terms, τ_L and τ_R are the characteristic times, and η, D, K, γ and δ are velocity constants.

Modulation by an output of the ultradian clock period T_{UR} is simulated by the introduction of a harmonic term as a forcing function into the slow equation, which then becomes:

$$\tau_L \frac{dL}{dt} = \eta - 2LR - DL + C \sin \Omega t \tag{13.2}$$

where

$$T_{UR} = 2\pi / \Omega << T_{\text{cell cycle}}$$

The auto-oscillating solution considers that cells divide when $L(t)$ reaches a threshold to initiate a rapid phase of the cycle.

13.5.3 Computer Simulations

Dynamic structure of the equations was studied numerically. The bifurcation diagram, a plot that indicates the dynamic state of the system as it is driven ever further into its nonlinear region (L_{max} vs. η, Fig. 13.4), shows that a chaotic region begins at η=4.65 and gives way to simple periodic oscillation at η=5.0: the route to chaos is via a quasiperiodic regime. A time-one map (R vs. L at time intervals of T_{UR}) has an intricate structure (not shown).

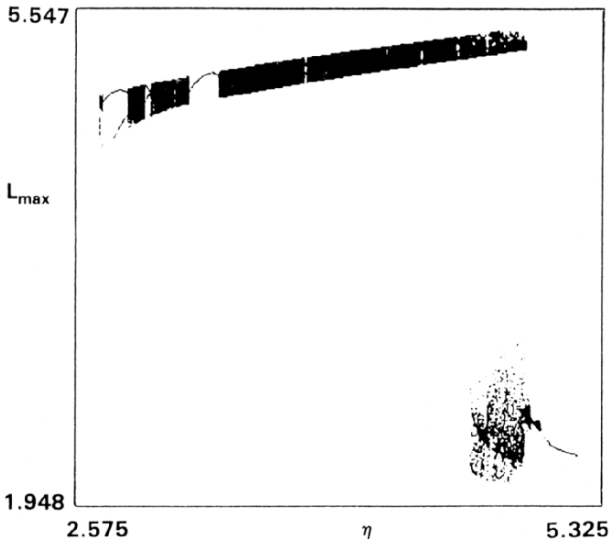

Fig. 13.4 Bifurcation diagram for the model described in Eqs. (13.1) and (13.2) as η varies between 2.5 and 5.2. For each value of η, 100 maxima of L are plotted following a transient of 100 time units. Other parameters are as follows: D=0.4, K=0.15, γ=1.5, δ=0.15, C=0.45, Ω=5.0, τ_L=1 and τ_R=0.1. The bifurcation diagram (L_{max} vs. η) shows that a chaotic region begins at η=4.65 and gives way to simple periodic oscillation at η=5.0: the route to chaos is via a quasi-periodic regime (reproduced with permission from Lloyd et al. 1992)

Calculation of the correlation dimension, D_2 (Grassberger and Procaccia 1983), gave a value of 1.95. Lyapunov exponents (Wolf et al. 1985), in bit per unit time, were λ_1=0.21, λ_2=0 and λ_3=–6.32, and the Lyapunov dimension was D_L=2.03. The positive value of λ_1 and the finite value for D_L confirm chaotic dynamics. A relative-frequency plot of cell cycle times for η=4.95 shows a trimodal distribution with negatively skewed dispersions (Fig. 13.5c): for comparison, also presented (Fig. 13.5a) are experimental data obtained by precise measurement of individual cell division times for the protozoon *Paramecium tetraurelia* (Lloyd and Kippert 1987), and (Fig. 13.5b) computer-simulated results for a noisy relaxation oscillator (Lloyd and Volkov 1990).

In the model presented here, dispersion and quantization of cell division times can arise as a consequence of the interaction of two oscillators (the cell cycle oscillator and the ultradian clock) in the absence of external noise. Other models have invoked the presence of a chaotic attractor in cell cycle dynamics (Engelberg 1968; Mackey 1985; Mackey et al. 1986; Grasman 1990; Crabb et al. 1996; Romond et al. 1999), but the present model is based on plausible biochemical processes (for the cell cycle oscillator) and an experimentally demonstrated intracellular timekeeper, the ultradian clock (Lloyd 1992). This results in a frequency distribution of cell division times that closely resembles those observed for animal cells (Klevecz 1976) and single-celled organisms

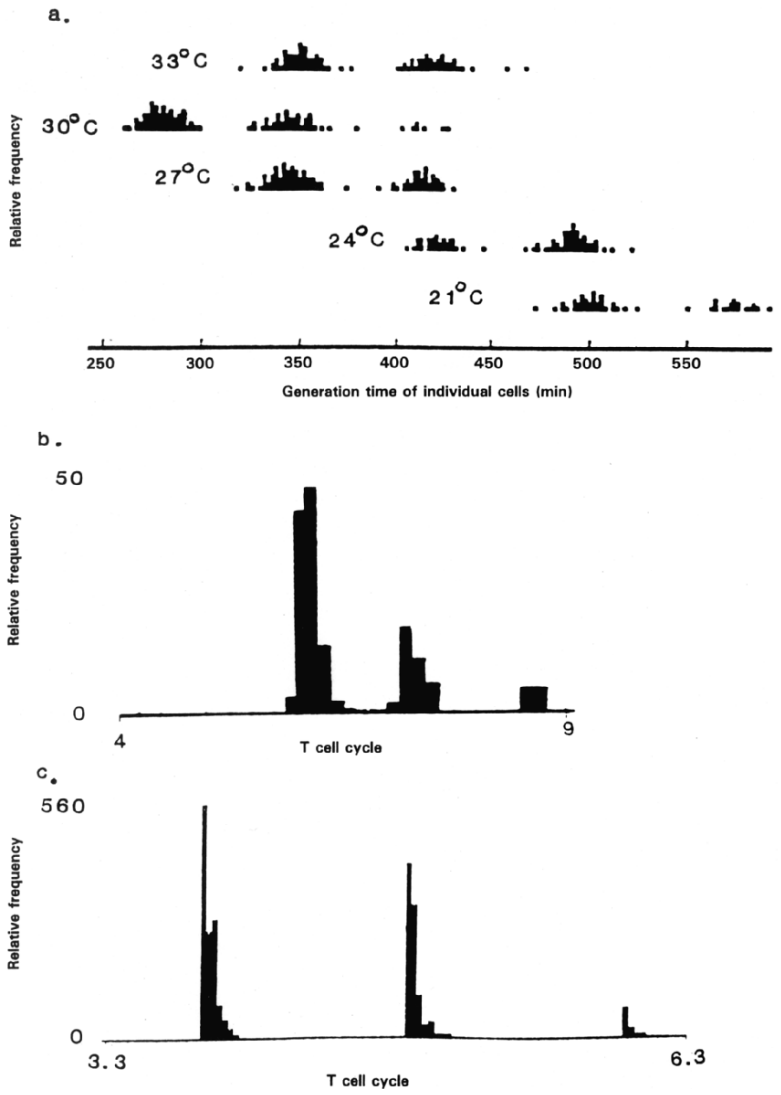

Fig. 13.5 Quantized cell cycle times. **a** Experimentally observed distributions of generation times of individual *Paramecium tetraurelia* cells at various steady-state growth temperatures. Each *square* represents timing of division of an isolated organism. **b** Generation time distributions from the model system incorporating noise (Eq. 13.2) when τ_L=1, τ_R=0.1, K=–0.15, D=0.4, γ=1.5, δ=0.15, Δ_η=0.3, ξ=random numbers (0,1), C=0.45, T_{UR}=1.25 and η=5.55. **c** Frequency distribution of cell cycle times ($T_{\text{cell cycle}}$) for parameter values as follows: η=4.95, D=0.4, K=0.15, γ=1.5, δ=0.15, C=0.45, Ω=5.0, τ_L=1 and τ_R=0.1 for Eq. (13.2) in the absence of a noise term (reproduced with permission from Lloyd and Rossi 1992)

(Lloyd and Kippert 1987). It remains to be established that evolutionary selection of chaotic cell division dynamics may have certain advantages not seen for noisy dynamics, e.g. robust defence against perturbations, ease of synchronous coupling of dynamics between individuals of a population or, in a multicellular tissue, construction of long period oscillations from high-frequency ones (Klevecz 1992). It seems likely that, in higher-plant systems, chaotic operation may have advantages for the optimization of water regulatory transport processes (Prytz 2001).

13.6 Chaos in Biochemistry and Physiology

Chaotic systems show some properties that could be exploited with oscillatory networks to enhance the "fitness" of living organisms. Physical and mathematical models that yield analogous properties have been studied extensively. Thus, Yang et al. (2000) have shown that smooth performance of the system state is typically achieved at higher period. Large networks of coupled oscillators undergo a transition to coherence (Restrepo et al. 2005), and the critical coupling strength can be determined (Baek and Ott 2004). Control of forced self-sustained oscillators can be achieved by delayed feedback in a weakly nonlinear van de Pol oscillator (Pyragiene and Pyragas 2005), and this principle can be utilized to stabilize the unstable periodic orbits of chaotic systems (Pyragas 2002; Fig. 13.6). Anticipated chaos is an unexpected consequence of a time delay between master and slave oscillators, where the latter can anticipate the former in a non-symmetric, coupled external-cavity-laser system (Rees et al. 2003). It seems likely that the intricate interweaving of time domains in living systems (Schibler and Naef 2005) may involve some of these fundamental and universal characteristics of complex systems that have evolved from simple networks (Goldbeter et al. 2001).

The identification of deterministic chaos in biochemical systems or in intact organisms is not straightforward (Yates 1992a, b; Hastings et al. 1993; A.L. Lloyd and Lloyd 1995; Aon et al. 2000). This is because the characterisation of a chaotic attractor requires as many as 10,000 data points. Furthermore, biological systems are subject to both internal and environmental noise as well as long-term drift; identification of chaos is thereby made more difficult. Computer simulations suggest that the living state (as modelled in silico) may be regarded as "being on the edge of chaos" (Langton 1991).

Olsen and Degn's (1977) demonstration of chaos in the peroxidase reaction in vitro has been repeatedly validated in "pseudo-open" (Geest et al. 1992) and in open systems (Hauck and Schneider 1994). Glycolytic chaos can be induced in glycolysis in cell-free systems prepared from yeast by periodic forcing with glucose (Markus et al. 1985; Nielsen et al. 1997). In vivo, a chaotic

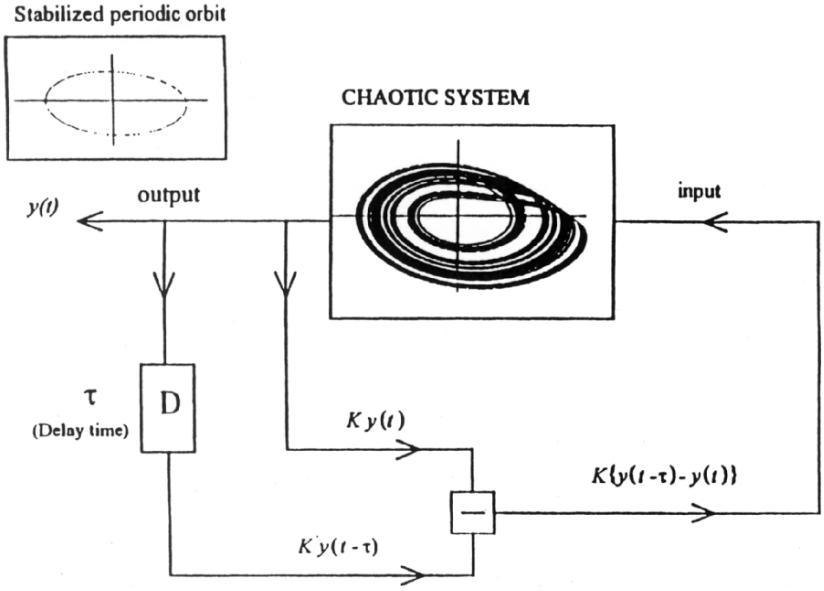

Fig. 13.6 Control of a chaotic system by feedback with delay to select a stable periodic orbit of required frequency (modified from Pyragas 2002)

growth regime has been demonstrated experimentally in a continuous culture of yeast (Davey et al. 1996), using a variety of time-series analyses (Fourier transformations, Hurst and Lyapunov exponents, the determination of embedding dimension and the Sugihara-May methodology for nonlinear time-series predictions).

The experimental work of D.B. Murray (cited in Lloyd et al. 2004) reveal that the presence of a strange attractor is strongly suggested as a feature of yeast respiratory dynamics in a continuous culture (Murray and Lloyd 2007). Lowering the pH of the culture from the value of 3.4 routinely employed in steps of 0.1 pH units drives the culture from its normally robust, regularly oscillating state (τ about 40 min; Lloyd and Murray 2000; Murray et al. 2003) into a chaotic state (Murray and Lloyd 2007; Fig. 13.7).

Experiments by M. Roussel and D. Lloyd (2007), using a membrane-inlet mass spectrometric probe to measure dissolved gases in a self-synchronous continuous culture of *S. cerevisiae*, provided further evidence for chaotic operation. Semi-continuous (data points obtained every 15 s) monitoring of dissolved O_2, CO_2 and H_2S over a period of 3 months gave 36,374 data points. When plotted, these suggest the presence of a strange attractor (Fig. 13.8): mathematical analysis is underway.

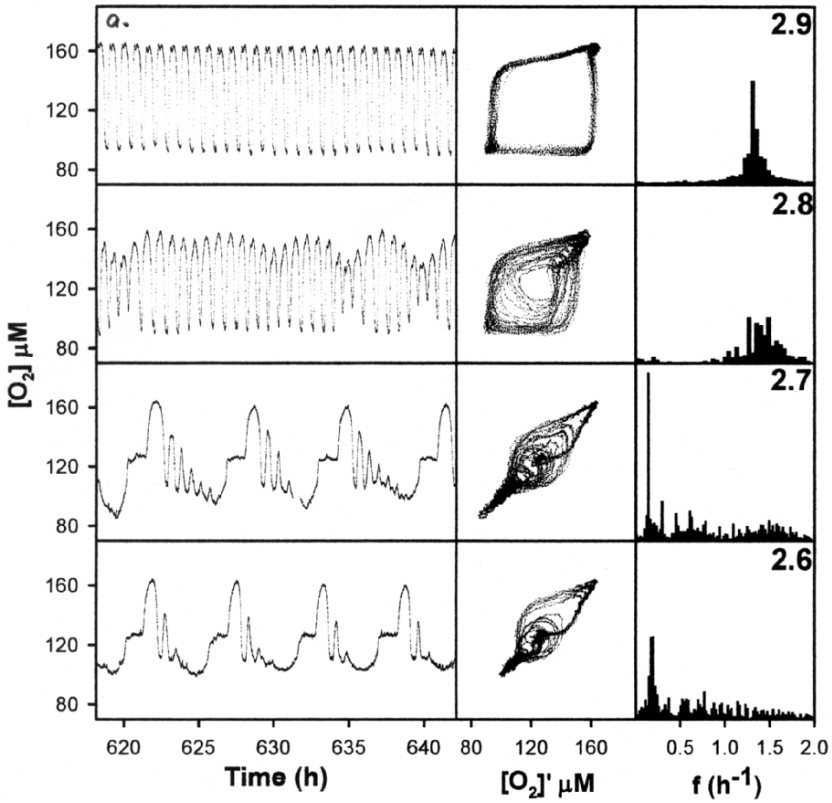

Fig. 13.7 Respiratory oscillations in a continuous culture of yeast: various outputs observed experimentally. In *a*, the 40-min ultradian clock is observed. Lowering the pH of the culture in steps of 0.1 pH unit drives the culture into a state where a low-dimensional chaotic attractor can be revealed (Murray and Lloyd 2007)

13.7 Functions of Rhythms

Table 13.2 summarises the possible uses to which rhythmic behaviour have been assigned. Autodynamic properties are necessary for the performance of cellular and molecular functions on each and every level of the spatiotemporal hierarchy. Steady-state operation is hardly possible in the dynamic whirlpool of life; even "structural" elements (cytoskeletal proteins) are being turned-over at rates that have been consistently underestimated. Homeodynamics (Yates 1992 a, b) ensures an apparent constancy of the structure–function continuum (Aon et al. 2000; Lloyd et al. 2001).

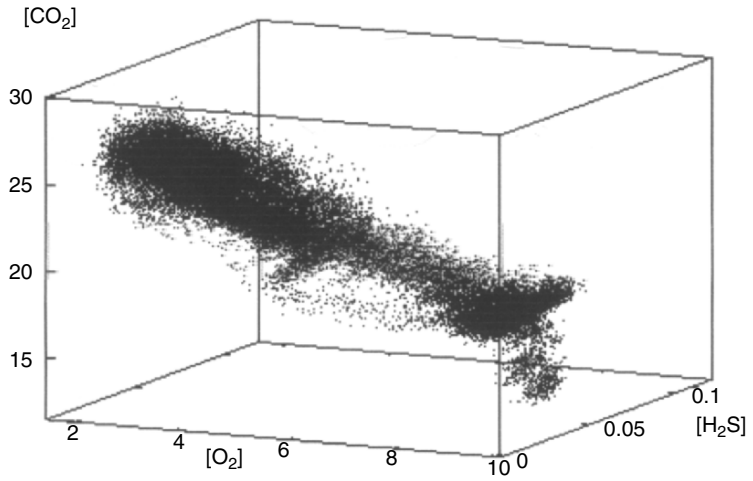

Fig. 13.8 A strange attractor in the dynamics of gas evolution/consumption in a continuously monitored, continuous culture of *S. cerevisiae*, as revealed by membrane inlet mass spectrometry. Values of *m*/*z* 32, 34 and 44 (O_2, H_2S and CO_2 respectively) were measured every 15 s, compared with *m*/*z* 40 (argon). The experiment lasted 3 months and 36,374 data points were collected (Roussel and Lloyd 2007)

Table 13.2 Functions of oscillations/rhythms/clocks

Number	Function
1	Increased energy efficiency? e.g. in glycolysis
2	Signalling, e.g. Ca^{2+} oscillator, O^{2-}. Amplitude modulation or frequency modulation
3	Spatial organization, e.g. in segmentation, the somite "clock"
4	Temporal organization a Matching intracellular/interorganism events with the environmental cycles, e.g. circadian rhythms, seasonal rhythms b Separating incompatible processes, e.g. photosynthesis producing oxygen vs. nitrogen fixation c Coordinating events in different compartments of the cell or of the organism, e.g. mitochondrion/nucleus – cytosol
5	Entrainment by a Phase b Frequency
6	Prediction (e.g. dawn or dusk by circadian rhythms)
7	Responsiveness
8	Biological "fitness"

13.8 Biological Functions of Chaotic Performance

The early idea that deterministic chaos is an indication of dysfunction in biological systems has been modified considerably and, although in some instances it may signify pathological state, there is now great interest in the possible advantages of chaotic performance in biology (Conrad 1986). Briefly, there are four spheres in which this topic has been considered: innovation, preservation of diversity, prevention of entrainment, and dissipation of disturbance (A.L. Lloyd and Lloyd 1995). A.L. Lloyd and Lloyd (1993) have further suggested that a controlled chaotic system may have another central and fundamental use: as the tuneable oscillator responsible for coordinated, integrated and coherent intracellular physiology.

Now that we realise that an infinite number of stable periodic orbits are embedded in the attractor (Ott et al. 1990) and that, in experimental systems, it is possible to select and stabilize each of these as required using control algorithms (Fig. 13.6; Pyragas 1992), it becomes clear that a highly adaptable and flexible dynamic system follows as a natural consequence (Lloyd and A.L. Lloyd 1994). Such a system could be of evolutionary advantage. A controlled chaotic attractor can ensure rapid switching between states, and thereby flexibility of response to changing conditions. Ultradian–circadian switching is one such example (A.L. Lloyd and Lloyd 1993, 1995). Self-optimization of dynamic behaviour and a robust defence of the status quo provide homeodynamics, rather than homeostasis (Yates 1992a, b; Lloyd 1994; Lloyd et al. 2001). The rich dynamic behaviour of life processes (relaxation times from (s to years) may stem from the outputs of controlled chaotic attractors. Yeast growing in continuous culture provides an excellent experimental system on which to demonstrate oscillatory and chaotic dynamics (Roussel and Lloyd 2007, Murray and Llyod 2007) of respiration (Figs. 13.7, 13.8) under controlled conditions of cell multiplication (Lloyd et al. 2003; Murray et al. 2003; Lloyd and Murray 2005).

Future work should include more detailed analysis of transcriptome expression and proteomic changes during the 40-min cycle to further characterize the intricacies of the oscillatory response and time-keeping functions that underlie cellular integrity. Interrogation of higher-frequency temporal coordination will require work with single organisms (Aon et al. 2007), rather than coherent populations, as higher time resolution will be necessary. This information will be crucial to a valid system-analysis approach to the temporal coherence of the organism.

13.9 Evolution of Rhythmic Performance

Self-sustaining oscillations have been studied in continuously fed chemical systems, and entrainment by temperature or light can be demonstrated. It is therefore quite conceivable that such systems may have developed on

prebiotic earth. In bacteria, control systems are ubiquitous and oscillatory states commonplace.

We may conjecture that systems that may have originated as somewhat "sloppy" control circuitry must have been exploited for all the known functions of biological oscillators (Table 13.2), and that a more precise and robust performance gradually evolved. Oscillations became rhythms, and the development of temperature compensation enabled timekeeping. This would have occurred on and between every timescale in the heterarchy of spatiotemporal organization, so that the highly interconnected network self-optimized to meet the demands of both external matching with geophysical cycles and internal coherence.

Thus, the multi-oscillator systems of present-day organisms (Lillo et al. 2001) consist not simply of the outputs of a small number of "canonical circadian genes" but rather of the entire metabolic circuitry as well as its translational and transcriptional networks.

The most dramatic step in evolution, the origin of eukaryotes (Carlisle 1980), has been the subject of much speculation. One model that now finds growing support is the acquisition of a sulphide-oxidizing α-proteobacterial symbiont by an Archaeal host that produced H_2S (Searcy 2003). The ultradian organization of the clock in yeast suggests that accommodation to this interrelationship involved separation in time of the reductive and oxidative stages thereby required (Lloyd and Murray 2006). I suggest that the ultradian clock preceded the circadian clock, and that the evolutionary development of the latter as a more recent phenomenon is echoed today in all higher organisms (Dutilleul et al. 2003). This relationship becomes most evident in those mutant organisms in which the disruption of circadian rhythms uncovers the underlying ultradians (Dowse and Ringo 1987; Lloyd 1998). One of the most pressing problems is to discover how the ca. 24-h rhythm was first constructed during evolution (and presumably is still being constructed) from higher-frequency ones: hypotheses abound for counters, couplers and rhythm generators (Lloyd et al. 1982b; Barrio et al. 1997; Fukuda et al. 2004; Paetkau et al. 2006). Good experiments are overdue. Those of Fuentes-Pardo and Sáenz (1988) with crayfish, and of Morré et al. (2002a, b) with plant and animal cultures indicate that alterations of the ultradian clock period produce commensurate alterations in circadian time. Ablation of circadian rhythmicity (by mutation or low temperature) reveals the underlying ultradian infrastructure. The circadian "clock genes" may be regarded as an integral part of a global oscillating system that involves many more control circuits than those studied to date in flies, moulds, mice and humans. Implications of these concepts for the differentiated organization of higher plants have recently been proposed (Lloyd 2006).

"We need to be extremely careful in not imposing our constructions on what exists. This becomes evident when we attempt to deal with multiple parallel processes going on in the same space ... The coherence of the system which may be impossible to define at the global level, is assuredly generated

by the properties of the elements, because the system exists and has survived the test of natural selection" (Brenner 1999).

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14 Modelling Ca^{2+} Oscillations in Plants

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Abstract

To interpret the physiological functions of Ca^{2+} oscillations in plant cells, one has to understand how Ca^{2+} oscillations are generated and how they are modified by internal as well as external stimuli. Yet, oscillations result from nonlinear interactions of different components, which makes it impossible to intuitively predict how a certain stimulus might affect the frequency or amplitude of a Ca^{2+} oscillation. Usually, it is not even possible to explain why certain conditions result in Ca^{2+} oscillations whereas others do not. The only way to try to *explain* intracellular Ca^{2+} oscillations is by a mathematical model. Here, we give an introduction on how such a mathematical model can be derived. To start with, a general scheme of the Ca^{2+} fluxes in a plant cell is translated into a set of simple differential equations. Using Sr^{2+}-induced Ca^{2+} oscillations in the unicellular green alga *Eremosphaera viridis* as an example, this general scheme is then developed into a complete mathematical model. For this, the different Ca^{2+} fluxes driving the Ca^{2+} oscillations are discussed and, term by term, integrated into two coupled, nonlinear differential equations. Where possible, the mathematical description of the transmembrane Ca^{2+} fluxes is based on experimental results obtained with *E. viridis*. In some cases, assumptions based on models of Ca^{2+} oscillations in animal cells are introduced. A couple of simplifications are made to prevent the mathematical model from becoming excessively complex. Despite these limitations, the derived mathematical model qualitatively reproduces the dose dependence of frequency and amplitude of Sr^{2+}-induced Ca^{2+} oscillations in *E. viridis*. Finally, it is discussed how Ca^{2+} oscillations in other plant cells – such as Nod factor-induced Ca^{2+} oscillations in root hairs or Ca^{2+} oscillations in stomatal guard cells – might be described mathematically.

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S. Mancuso and S. Shabala (Eds.)
Rhythms in Plants: Phenomenology, Mechanisms, and Adaptive Significance

14.1 Introduction

Being a result of nonlinear interactions among different components of a dynamic system, oscillations can hardly be understood intuitively. When it comes to sustained oscillations of the cytosolic free Ca^{2+} concentration – or Ca^{2+} oscillations for short – it is obvious that Ca^{2+} channels and Ca^{2+} pumps are involved (Berridge 1997; Hetherington and Brownlee 2004). Ca^{2+} channels mediate the influx of Ca^{2+} into the cytosol, from the exterior or from intracellular Ca^{2+} stores, and Ca^{2+} pumps mediate Ca^{2+} efflux from the cytosol, restoring cytosolic Ca^{2+} levels and refilling intracellular stores (Fig. 14.1). In such a generalised model, Ca^{2+} pumps can be understood as any type of membrane protein that transports Ca^{2+} against its electrochemical potential gradient. From a scheme such as that given in Fig. 14.1, however, it is impossible to predict under which conditions Ca^{2+} oscillations might be observed or not. Only a mathematical model can assess whether a certain system will show stable oscillations (i.e. limit cycle oscillations, see below) and under which conditions.

In animal cells, attempts to describe Ca^{2+} oscillations by mathematical models have formed an integral part of this research sphere almost from the very beginning (Meyer and Stryer 1988; Dupont et al. 1990), and the resulting mathematical models significantly contributed to a better understanding and the design of more refined experiments. By contrast, in plant cells we are

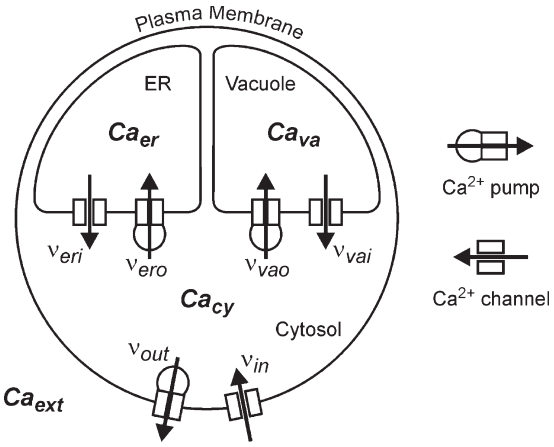

Fig. 14.1 General scheme of Ca^{2+} concentrations and Ca^{2+} fluxes in a plant cell. Ca_{cy}, Ca_{er}, Ca_{va} and Ca_{ext} are cytosolic, ER, vacuolar and external Ca^{2+} concentration respectively; v_{in} is influx of Ca^{2+} across the plasma membrane, v_{out} is transport of Ca^{2+} out of the cell, v_{eri} is influx of Ca^{2+} into the cytosol from the ER, v_{ero} is transport of Ca^{2+} out of the cytosol into the ER, v_{vai} is influx of Ca^{2+} into the cytosol from the vacuole, and v_{vao} is transport of Ca^{2+} out of the cytosol into the vacuole. Here, a Ca^{2+} pumps can be any membrane transport molecule that transports Ca^{2+} against its electrochemical potential gradient (i.e. out of the cytosol), and a Ca^{2+} channel can be any transport process that enables Ca^{2+} to cross the membrane down its electrochemical potential gradient

aware of only a single attempt to model Ca^{2+} oscillations mathematically (Bauer et al. 1998a). Oscillations in plant membrane potential resulting from fluctuations in membrane transporter activity have been described mathematically (Gradmann 2001; Shabala et al. 2006) but no mathematical models have been developed yet for Ca^{2+} oscillations observed in stomatal guard cells, pollen tubes or legume root hairs.

Mathematically, Ca^{2+} oscillations can be described at two different levels – on a molecular level or on a compartment level. On a molecular level, the spatial and temporal distribution of discrete Ca^{2+} ions is calculated by stochastic algorithms (Kraus et al. 1996; Kummer et al. 2005). On a compartment level, continuous concentrations are calculated by integrating differential equations. This latter deterministic approach assumes homogeneous concentration within each compartment, and is mathematically much simpler than that for stochastic models. In small compartments, however, particle numbers may become so low that spatial fluctuations can no longer be ignored, and the assumption of homogeneous concentrations is no longer valid. For example, a compartment of 1 μm^3 with a free Ca^{2+} concentration of 100 nM contains only 60 free Ca^{2+} cations. Compared to this, the cytosolic volume of an *Arabidopsis* guard cell is in the order of 10 μm^3, and cell organelles may have volumes of less than 1 μm^3. Even though there are indications that stochastic effects might be relevant at physiological Ca^{2+} concentrations (Kummer et al. 2005), to date most mathematical models of Ca^{2+} oscillations are based on deterministic models (Schuster et al. 2002). The following example illustrating how a mathematical model of Ca^{2+} oscillations can be developed is based on experiments performed with the unicellular green alga *Eremosphaera viridis*. The spherical cells of *E. viridis* have a diameter of about 150 µm, resulting in compartment volumes that most likely justify a deterministic approach.

For the benefit of the reader, in the following are listed the definitions of some key abbreviations used in this chapter. CICR, Ca^{2+}-induced Ca^{2+} release; CPA, cyclopiazonic acid; DBHQ, 2,5-di-tert-butylhydroquinone; IP_3, inositol 1,4,5-trisphosphate.

14.2 Developing a Mathematical Model

To understand how a mathematical model of Ca^{2+} oscillations can be developed, let us start with the general scheme in Fig. 14.1, summarizing Ca^{2+} concentrations and Ca^{2+} fluxes in a plant cell. For this general model, the differential equations describing changes of intracellular Ca^{2+} concentrations can directly be written as:

$$\frac{\mathrm{d}}{\mathrm{d}t} Ca_{\mathrm{cy}} = \nu_{\mathrm{in}} - \nu_{\mathrm{out}} + \nu_{\mathrm{eri}} - \nu_{\mathrm{ero}} + \nu_{\mathrm{vai}} - \nu_{\mathrm{vao}} \tag{14.1}$$

$$\frac{\mathrm{d}}{\mathrm{d}t} Ca_{\mathrm{er}} = \rho_{\mathrm{er}} (\nu_{\mathrm{ero}} - \nu_{\mathrm{eri}}) \tag{14.2}$$

$$\frac{\mathrm{d}}{\mathrm{d}t} Ca_{\mathrm{va}} = \rho_{\mathrm{va}} (\nu_{\mathrm{vao}} - \nu_{\mathrm{vai}}) \tag{14.3}$$

where Ca_{cy}, Ca_{er}, Ca_{va} and Ca_{ext} are cytosolic, ER, vacuolar and external Ca^{2+} concentration respectively, ν_{in} is influx of Ca^{2+} across the plasma membrane, ν_{out} is transport of Ca^{2+} out of the cell, ν_{eri} is influx of Ca^{2+} into the cytosol from the ER, ν_{ero} is transport of Ca^{2+} out of the cytosol into the ER, ν_{vai} is influx of Ca^{2+} into the cytosol from the vacuole, ν_{vao} is transport of Ca^{2+} out of the cytosol into the vacuole, and ρ_{er} and ρ_{va} are the cytosol/ER and cytosol/vacuole volume ratios respectively. Ca_{ext}, the external Ca^{2+} concentration, is usually assumed to be constant. Throughout this chapter, italic symbols of substances are used for concentrations, e.g. $[Ca^{2+}]_{\mathrm{cy}} \rightarrow Ca_{\mathrm{cy}}$.

Obviously, the general model presented in Fig. 14.1 and Eqs. (14.1) to (14.3) is grossly simplified. It contains only two intracellular Ca^{2+} stores, does not include any signalling components such as IP_3, and neither is Ca^{2+} buffering considered. It would indeed be possible to add additional Ca^{2+} stores but, as demonstrated below, a single Ca^{2+} store is sufficient for the generation and maintenance of stable Ca^{2+} oscillations. Signalling components such as IP_3, which release Ca^{2+} from internal stores, are an important component of most models for Ca^{2+} oscillation in animal cells (Schuster et al. 2002). In a less complex case, a certain concentration of IP_3 promotes Ca^{2+} release from internal stores into the cytoplasm. In the general scheme given above, this would simply cause an increase in ν_{eri} and/or ν_{vai}. In more complex cases, IP_3 (the IP_3 concentration) may be a separate variable, and IP_3 dynamics is described by a differential equation. Ca^{2+} binding by buffers can modulate Ca^{2+} oscillations, and mathematical models have been developed that describe Ca^{2+} binding by a separate variable/differential equation. To keep things simple, we stay with the general model presented in Fig. 14.1 and Eqs. (14.1) to (14.3). It contains (almost) everything needed for stable cytosolic Ca^{2+} oscillations.

To start with, we consider an example of Ca^{2+} oscillations observed in a plant cell. When the unicellular green alga *E. viridis* is perfused with a bath solution containing Sr^{2+} (0.1 mM or more) or caffeine (1 mM or more), cytosolic Ca^{2+} oscillations are observed (Bauer et al. 1997). Our group performed a pharmacological characterization of these Ca^{2+} oscillations to elucidate the molecular mechanisms of Ca^{2+} oscillations in a plant cell. Based on experimental results, we were able to develop a mathematical model that describes Sr^{2+}-induced Ca^{2+} oscillations in *E. viridis* (Bauer et al. 1998a). The principal findings of the pharmacological experiments are summarized in Fig. 14.2. In the following sections, these results are used to develop Eqs. (14.1) and (14.2) into a mathematical model for Sr^{2+}-induced Ca^{2+} oscillations.

We begin at the plasma membrane (ν_{in} and ν_{out} in Fig. 14.1). Models of Ca^{2+} oscillations can be divided into two groups – those with constant total cellular Ca^{2+} content (ν_{in}=0, ν_{out}=0) and those with changing total cellular

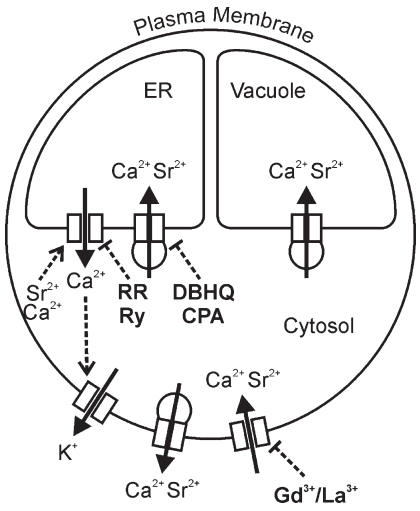

Fig. 14.2 Model for Sr^{2+}-induced Ca^{2+} oscillations in the unicellular green alga *Eremosphaera viridis*. Sr^{2+} enters the cell via plasma membrane cation channels that are blocked by Gd^{3+} or La^{3+}. Most of the Sr^{2+} taken up into the cell is compartmentalized into the vacuole. Ca^{2+} (and Sr^{2+}?) is transported into the ER by Ca^{2+}-ATPases that are specifically blocked by DCBQ and CPA. In the cytosol, Ca^{2+} and Sr^{2+} induce a Ca^{2+}-induced Ca^{2+} release (CICR) from the ER. The ER Ca^{2+} channel is blocked by either ruthenium red (RR) or ryanodine (Ry). Increasing cytosolic Ca^{2+} causes the opening of plasma membrane K^+ channels, resulting in a hyperpolarization (Bauer et al. 1998a)

Ca^{2+} content, due to Ca^{2+} fluxes across the plasma membrane. To test for the importance of Ca^{2+} uptake into the cell, we applied La^{3+} and Gd^{3+} during Sr^{2+}-induced or caffeine-induced Ca^{2+} oscillations. Both trivalent cations rapidly and reversibly blocked Ca^{2+} oscillations induced by either Sr^{2+} or caffeine (Bauer et al. 1997, 1998a). Cation uptake measurements (by ICP-AES analysis of cell sap) showed that neither La^{3+} nor Gd^{3+} reached detectable intracellular concentrations. Therefore, La^{3+} and Gd^{3+} most likely inhibited Ca^{2+} oscillations by blocking Ca^{2+} influx across the plasma membrane, indicating the necessity of Ca^{2+} uptake into the cell for sustained Ca^{2+} oscillations.

Ca^{2+} influx across the plasma membrane, v_{in}, depends on Ca_{ext} and the electrochemical potential difference for Ca^{2+}. Ca_{ext} was constant under our experimental conditions and, therefore, does not need further consideration. The electrochemical potential gradient for Ca^{2+}, however, does change during Ca^{2+} oscillations. Increasing Ca_{cy} during a Ca^{2+} spike decreases the chemical potential difference for Ca^{2+} across the plasma membrane. Moreover, Ca^{2+} oscillations in *E. viridis* have been shown to be accompanied by membrane potential oscillations, caused by the Ca^{2+}-dependent opening of plasma membrane K^+ channels (Bauer et al. 1998b). Each cytosolic Ca^{2+} spike induces a hyperpolarization increasing the electrical potential difference. How do these

opposing changes in chemical and electrical driving force affect Ca^{2+} influx into the cell (v_{in})? To answer this question, we systematically varied the electrical driving force, i.e. the membrane potential, during Sr^{2+}-induced Ca^{2+} oscillations. When the external K^+ concentration is increased, the membrane potential of *E. viridis* becomes less negative and the transient hyperpolarizations (at 0.1 mM external K^+) accompanying Ca^{2+} spikes change into transient depolarizations (at 100 mM external K^+). It turns out that, cytosolic Ca^{2+} oscillations are not significantly altered by the membrane potential change induced by increasing external K^+ (Bauer et al. 1998a). Even though Ca^{2+} oscillations in *E. viridis* are accompanied by membrane potential oscillations, the membrane potential does not seem to have a large effect on the Ca^{2+} oscillations. For an initial, simplified mathematical model, therefore, we assumed v_{in}=const.

If there is Ca^{2+} uptake into the cell, then there has to be Ca^{2+} transport out of the cell to prevent Ca^{2+} from accumulating over time. For the mathematical model, we simply assume the activity of a plasma membrane Ca^{2+}-ATPase that pumps Ca^{2+} out of the cell with a rate proportional to the cytosolic concentration, Ca_{cy} ($v_{out}=k_{out}\cdot Ca_{cy}$). This assumption, again, is a simplification, ignoring saturation of transport rates at high substrate concentrations, as described by Michaelis-Menten-type transport kinetics. Yet, many transporters operate at substrate concentrations below their K_m (Michaelis constant) values, resulting in an effectively linear relationship between transport rate and substrate concentration. Even if a linear relationship is not exactly given, this approximation seems to be justified for qualitative description.

To describe self-sustained oscillations by a system of differential equations, at least two variables are needed (Stucki and Somogyi 1994). In the case of Ca^{2+} fluxes between the cytosol and the intracellular Ca^{2+} stores (v_{eri}, v_{ero}, v_{vai}, v_{vao}), one of these variables is obviously Ca_{cy}, the cytosolic Ca^{2+} concentration; the other variable might be the Ca^{2+} concentration inside an intracellular Ca^{2+} store, Ca_{er} or Ca_{va} (in the general model given in Fig. 14.1 and Eqs. 14.1 to 14.3). Alternatively, the other variable might be IP_3 (or the changing sensitivity of IP_3 receptors), or the number of Ca^{2+} buffer groups binding Ca^{2+}, or the membrane potential (Schuster et al. 2002). More complex models may contain more than two variables.

In the case of Sr^{2+}-induced (or caffeine-induced) Ca^{2+} oscillations in *E. viridis*, there is experimental evidence that the Ca^{2+} concentration of an intracellular store – most likely the ER (Ca_{er}) – is such another variable. Why the ER and not the vacuole? The Ca^{2+} content of the vacuole is probably too high to show significant changes during cytosolic Ca^{2+} oscillations. In *E. viridis*, the vacuolar free Ca^{2+} concentration is 200 µM, compared to 160 nM in the cytosol (Bethmann et al. 1995). Based on a roughly tenfold larger volume of the vacuole, the total Ca^{2+} content of the vacuole would be four orders of magnitude higher than that of the cytosol – assuming comparable Ca^{2+} buffer capacities. Even if the cytosolic Ca^{2+} buffer capacity were 100 times larger than the vacuolar buffer capacity, which is not likely to be the case (Schönknecht

and Bethmann 1998), a Ca^{2+} release from the vacuole that increases the cytosolic free Ca^{2+} concentration from 160 nm to 1.6 µM would not result in a significant change of the free vacuolar Ca^{2+} concentration. It is the large amount of Ca^{2+} accumulated inside the vacuole that makes is rather unlikely that cytosolic Ca^{2+} oscillations are accompanied by vacuolar Ca^{2+} oscillations, thus ruling out the vacuolar Ca^{2+} concentration (Ca_{va}) as second variable for the mathematical model.

In line with these quantitative considerations, our experimental evidence points to a rather small intracellular Ca^{2+} store driving Sr^{2+}-induced (and caffeine-induced) Ca^{2+} oscillations in *E. viridis*. Application of the ER Ca^{2+}-ATPase blocker DBHQ or CPA (10 µM in each case) stops Sr^{2+}-induced Ca^{2+} oscillations within a few minutes. The baseline Ca_{cy} level and Sr^{2+} uptake and compartmentalization are not affected by DBHQ, indicating that Ca^{2+} and Sr^{2+} homeostasis is still maintained by Ca^{2+} pumps not inhibited by DBHQ (Bauer et al. 1998a). DBHQ and CPA have been shown to act specifically on ER Ca^{2+}-ATPases both in animal (Inesi and Sagara 1994) and plant cells (Logan and Venis 1995; Hwang et al. 1997; Liang et al. 1997). As evident from Fig. 14.2, blocking ER Ca^{2+}-ATPases by DBHQ or CPA prevents the reuptake of Ca^{2+} into the ER, resulting in a depletion of the intracellular Ca^{2+} store that is needed to drive the Ca^{2+} oscillations. For the mathematical model, we assume the transport rate of the ER Ca^{2+}-ATPase to be proportional to the cytosolic Ca^{2+} concentration ($v_{ero}=k_{ero}\cdot Ca_{cy}$) – as we assume for the plasma membrane Ca^{2+}-ATPase, (v_{out}, see above).

Finally, we need a mathematical term describing the Ca^{2+} efflux from the intracellular store (v_{eri}) that increases Ca_{cy} during Ca^{2+} oscillations. Most Ca^{2+} oscillations investigated in animal cells seem to be driven by intracellular Ca^{2+} release via IP_3 receptors (Schuster et al. 2002). TMB_8 (3,4,5-trimethoxybenzoic acid 8-diethylaminooctyl ester), an inhibitor of IP_3-induced Ca^{2+} release in *E. viridis* (Förster 1990), does not block Sr^{2+}-induced Ca^{2+} oscillations at concentrations of up to 200 µM, indicating that IP_3 receptors are not involved. In animal cells, caffeine and Sr^{2+} are known to induce Ca^{2+} release from the ER (and sarcoplasmic reticulum) via the ryanodine receptor (Meissner 1994). In *E. viridis*, both Sr^{2+} and caffeine induce identical Ca^{2+} oscillations (Bauer et al. 1997). Ruthenium red (Ma 1993) and ryanodine (Smith et al. 1988) are known to be specific for the ryanodine receptor in animal cells (Ehrlich et al. 1994), and have been shown to block Ca^{2+} release in plant cells (Allen et al. 1995; Muir and Sanders 1996). When microinjected into *E. viridis*, both inhibitors block Sr^{2+}-induced (and caffeine-induced) membrane potential oscillations accompanying Ca^{2+} oscillations, whereas membrane potential changes induced by other changes (such as darkening) are not affected (Bauer et al. 1998a). This indicates that ruthenium red and ryanodine specifically block Sr^{2+}-induced Ca^{2+} release from the ER but not the Ca^{2+} release from chloroplasts after darkening. Summarizing, in *E. viridis* cytosolic Sr^{2+} seems to induce Ca^{2+} release from the ER by activating a ryanodine receptor type of Ca^{2+} channel (see Fig. 14.2).

How can this component, the Sr^{2+}-induced Ca^{2+} release from the ER (v_{eri}), be mathematically described to complete the mathematical model of Sr^{2+}-induced Ca^{2+} oscillations in *E. viridis*? Very little is known about ryanodine receptor-type Ca^{2+} channels in plants. At this point, to achieve a qualitative description of the Sr^{2+}-induced Ca^{2+} release in *E. viridis*, one has to rely on data from animal cells. Ryanodine receptors, similarly to IP_3 receptors, show so-called Ca^{2+}-induced Ca^{2+} release (CICR). This means that a relatively small initial increase in Ca_{cy} activates ryanodine receptors, resulting in Ca^{2+} release and, thus, a further increase of Ca_{cy}. Almost all models of Ca^{2+} oscillations with only two variables are based on CICR as a positive feedback element (Schuster et al. 2002). This is important, since another requirement for stable oscillations is feedback. At least one feedback step has to connect the variables. This feedback is usually positive, since negative feedback loops in most cases act as stabilizing elements, returning the variables to their steady-state values preventing oscillations (Stucki and Somogyi 1994).

CICR is a nonlinear process and, in mathematical models of Ca^{2+} oscillations, cooperative Ca^{2+} binding (with Hill coefficients of two or higher) to the ryanodine receptor or IP_3 receptor is often assumed (Somogyi and Stucki 1991; Schuster et al. 2002). For isolated ryanodine receptor complexes of the sacroplasmic reticulum, a positive cooperativity for Ca^{2+}-dependent channel gating has been shown (Meissner et al. 1986; Pessah et al. 1987). The positive nonlinear feedback by CICR is a central element of many Ca^{2+} oscillation models (Berridge 1990; Dupont and Goldbeter 1993; Bootman et al. 1996). Why is nonlinear feedback important? A further requirement for stable oscillations is 'sufficient total nonlinearity' (Stucki and Somogyi 1994), as a system of linear kinetics will not give rise to stable oscillations. In most models, it is the feedback element that is assumed to be nonlinear. This nonlinearity can come from Michaelis-Menten-type kinetics, positive cooperativity, etc. (Stucki and Somogyi 1994). In line with existing models of Ca^{2+} oscillations and what is known about the ryanodine receptor in animal cells, we assume that Ca^{2+}-induced Ca^{2+} release from the ER (v_{eri}) in *E. viridis* is based on cooperative Ca^{2+} binding. This is mathematically formulated as:

$$v_{eri} = k_{eri} \cdot \frac{Ca_{cy}^{\,n}}{K^n + Ca_{cy}^{\,n}} \cdot (Ca_{er} - Ca_{cy}) \qquad (14.4)$$

where k_{eri} is the rate constant for Ca^{2+} release by the ryanodine receptor, K is the affinity constant for Ca^{2+} binding (half maximum binding), n is the Hill coefficient, and all other symbols are as in the general model given in Fig. 14.1 and Eqs. (14.1) to (14.3). Equation (14.4) describes a Ca^{2+} channel (k_{eri}) that is modulated in a cooperative manner (K, n) by cytosolic Ca^{2+} (Ca_{cy}) while the difference in Ca^{2+} concentration between ER and cytosol (Ca_{er}-Ca_{cy}) drives the Ca^{2+} flux (v_{eri}).

Next, Sr^{2+} has to be included. It is known that Sr^{2+}, similarly to Ca^{2+} (and caffeine), gates the ryanodine receptor open, triggering Ca^{2+} release

(Meissner 1994). For Sr^{2+}-induced Ca^{2+} oscillations in *E. viridis*, it is therefore assumed that there is both a Ca^{2+}-induced Ca^{2+} release and a Sr^{2+}-induced Ca^{2+} release from the ER via a ryanodine receptor-type Ca^{2+} channel. Accordingly, Eq. (14.4) is extended to:

$$\nu_{\mathrm{eri}} = k_{\mathrm{eri}} \cdot \frac{(Ca_{\mathrm{cy}} + Sr_{\mathrm{cy}})^n}{K^n + (Ca_{\mathrm{cy}} + Sr_{\mathrm{cy}})^n} \cdot (Ca_{\mathrm{er}} - Ca_{\mathrm{cy}}) \tag{14.5}$$

where Sr_{cy} is the cytosolic Sr^{2+} concentration. For simplicity, the same affinity constant, K, and Hill coefficient, n, are assumed for Ca^{2+} and Sr^{2+}.

Equation (14.5) describes a sigmoid function with a very low Ca^{2+} flux, ν_{eri}, in the absence of Sr^{2+} (Sr_{cy}=0) and at low Ca_{cy} (<200 nM at rest). However, we have experimental evidence that there is a considerable Ca^{2+} efflux from the ER even in the absence of Sr^{2+} and at low Ca_{cy}. Incubation of *E. viridis* with the ER Ca^{2+}-ATPase blocker DBHQ or CPA for more than 5 min completely inhibits Sr^{2+}-induced Ca^{2+} oscillations (Bauer et al. 1998a). Similarly to animal cells (Kass et al. 1989; Demaurex et al. 1992), there probably is a continuous efflux of Ca^{2+} from the ER in *E. viridis* and, when the compensating Ca^{2+} uptake by the ER Ca^{2+}-ATPase is inhibited, this results in store depletion preventing Ca^{2+} oscillations. To account for this continuous Ca^{2+} efflux, an additional 'leak' term, $k_{\mathrm{erL}} \cdot (Ca_{\mathrm{er}} - Ca_{\mathrm{cy}})$, is introduced that describes a Ca^{2+} efflux proportional to the driving force – the difference in Ca^{2+} concentration between the ER and cytosol ($Ca_{\mathrm{er}} - Ca_{\mathrm{cy}}$). This leak term is added to Eq. (14.5) to obtain a complete description of the Ca^{2+} efflux from the ER (ν_{eri}):

$$\nu_{\mathrm{eri}} = \left(k_{\mathrm{erL}} + k_{\mathrm{eri}} \cdot \frac{\left(Ca_{\mathrm{cy}} + Sr_{\mathrm{cy}}\right)^n}{K^n + \left(Ca_{\mathrm{cy}} + Sr_{\mathrm{cy}}\right)^n} \right) \cdot \left(Ca_{\mathrm{er}} - Ca_{\mathrm{cy}}\right) \tag{14.6}$$

where k_{erL} is the rate constant (proportionality factor) for the Ca^{2+} leak from the ER. Having discussed all Ca^{2+} fluxes that are summarized in the general model in Fig. 14.1 and Eqs. (14.1) to (14.3), ν_{out}, ν_{eri} and ν_{ero} can now be substituted, converting Eqs. (14.1) and (14.2) into:

$$\begin{cases} \dfrac{\mathrm{d}}{\mathrm{d}t} Ca_{\mathrm{cy}} = \nu_{\mathrm{in}} - \left(k_{\mathrm{out}} + k_{\mathrm{ero}}\right) \cdot Ca_{\mathrm{cy}} + \left(k_{\mathrm{erL}} + k_{\mathrm{eri}} \cdot \dfrac{\left(Ca_{\mathrm{cy}} + Sr_{\mathrm{cy}}\right)^n}{K^n + \left(Ca_{\mathrm{cy}} + Sr_{\mathrm{cy}}\right)^n} \right) \left(Ca_{\mathrm{er}} - Ca_{\mathrm{cy}}\right) \cdot \\ \dfrac{\mathrm{d}}{\mathrm{d}t} Ca_{\mathrm{er}} = \rho_{\mathrm{er}} \left(k_{\mathrm{ero}} \cdot Ca_{\mathrm{cy}} - \left(k_{\mathrm{erL}} + k_{\mathrm{eri}} \cdot \dfrac{(Ca_{\mathrm{cy}} + Sr_{\mathrm{cy}})^n}{K^n + (Ca_{\mathrm{cy}} + Sr_{\mathrm{cy}})^n} \right) \cdot \left(Ca_{\mathrm{er}} - Ca_{\mathrm{cy}}\right) \right) \end{cases} \tag{14.7}$$

Figure 14.3 gives a scheme explaining Eq. (14.7). Cytosolic Sr^{2+} initializes Ca^{2+}/Sr^{2+}-induced Ca^{2+} release from the ER. Due to a nonlinear positive feedback (Eq. 14.5), this results in a rapid, autocatalytic increase of Ca_{cy}.

Decreasing Ca_{er} and increasing Ca_{cy} slow down Ca^{2+} release. The ER Ca^{2+}-ATPase (k_{ero}) refills the ER Ca^{2+} store ($Ca_{er}\uparrow$) and, together with the plasma membrane Ca^{2+}-ATPase (k_{out}), causes Ca_{cy} to decrease to the baseline level once again.

14.3 Discussion of the Model

A comparison of Figs. 14.1 and 14.2 with Fig. 14.3 shows that the vacuole is not included in the mathematical model. The quantitative considerations given above indicate that Ca_{va} is unlikely to oscillate in *E. viridis*. In addition, there is no experimental evidence that Ca^{2+} fluxes between the cytosol and the vacuole play a significant role for Sr^{2+}-induced Ca^{2+} oscillations in *E. viridis*. It therefore seems justified to omit Ca_{va}, v_{vai} and v_{vao} in the mathematical model (Eq. 14.7).

The differential equations shown in Eq. (14.7) can not be integrated analytically but only numerically, i.e. step by step, starting with a certain set of

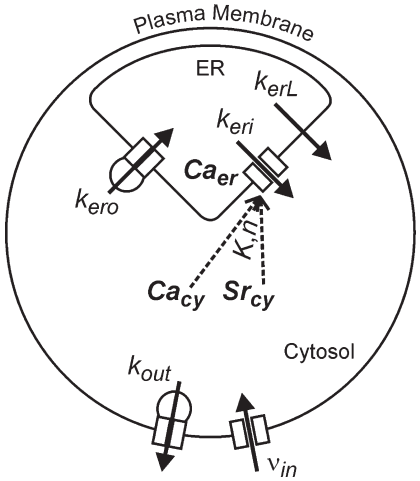

Fig. 14.3 Model of the Ca^{2+} oscillator described by Eq. (14.7). All parameters and variables used in Eq. (14.7) for the mathematical model of Sr^{2+}-induced Ca^{2+} oscillations in *E. viridis* are depicted in a schematic way. There is a constant Ca^{2+} influx from the external medium (v_{in}), and this constant Ca^{2+} influx is compensated by a plasma membrane Ca^{2+}-ATPase that pumps Ca^{2+} out of the cytosol (k_{out}). The ER is the intracellular Ca^{2+} store driving the Ca^{2+} oscillations, and Ca_{er} – in addition to Ca_{cy} – is the second variable changing over time in this model. There is a continuous efflux of Ca^{2+} from the ER due to a leak (k_{erL}). Sr^{2+} most likely enters the cell by the same pathway as does Ca^{2+}, and a Ca^{2+}/Sr^{2+}-induced Ca^{2+} release from the ER (k_{eri}) causes the rapid increase of Ca_{cy} initializing Ca^{2+} oscillations. The binding of Ca^{2+} and Sr^{2+} to the ER Ca^{2+} release channel (ryanodine receptor type) is assumed to be cooperative (K, n). The Ca^{2+} store is refilled by a ER Ca^{2+}-ATPase (k_{ero})

start values, $Ca_{cy}(t=0)$ and $Ca_{er}(t=0)$. This can be conveniently performed using Mathematica© (Wolfram Research Inc., Champaign, IL) or any other comparable software package. Figure 14.4 compares Sr^{2+}-induced Ca^{2+} oscillations measured in *E. viridis* (Fig. 14.4a) with Ca^{2+} oscillations calculated by numerically solving Eq. (14.7), the latter shown in Fig. 14.4b. In Fig. 14.4b, the following parameter set was used: $\nu_{in}=1$, $k_{out}=1$, $k_{ero}=2$, $k_{erL}=0.05$, $k_{eri}=1.2$, $K=2.5$, $n=4$, $Sr_{cy}=0.1$, $\rho_{er}=1$, with $Ca_{cy}(t=0)=0.2$ and $Ca_{er}(t=0)=24$ as start values. The same parameter set, but with $Sr_{cy}=0$, does not result in oscillations – as should be the case for Sr^{2+}-induced Ca^{2+} oscillations.

As a simplification, no units are given for the parameter values used to solve Eq. (14.7); as a result, the axes for the calculated Ca^{2+} oscillations (Fig. 14.4 b and c) are given in arbitrary units. For a first qualitative evaluation, this seems justified. For a more detailed simulation yielding time axes in seconds

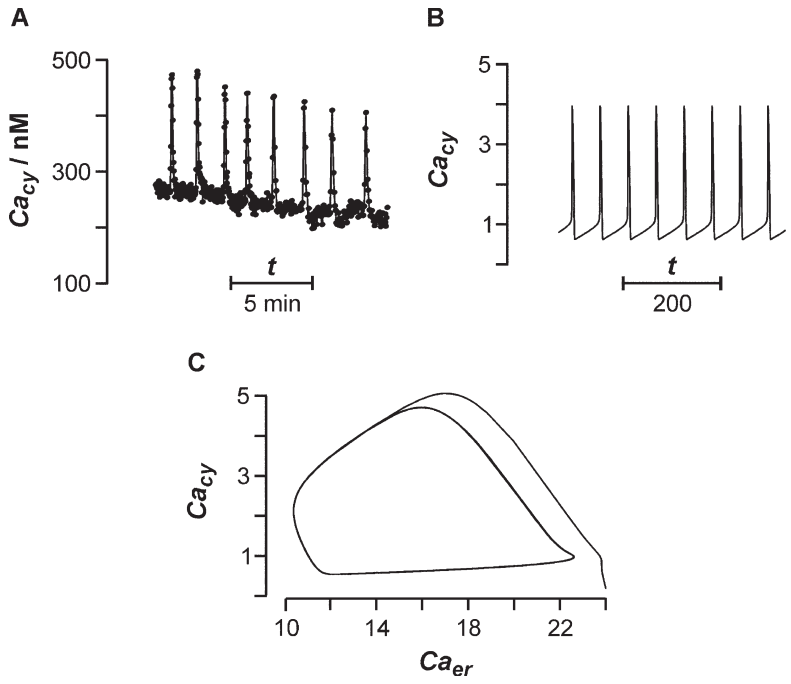

Fig. 14.4 Measured and calculated Ca^{2+} oscillations. **a** Sr^{2+}-induced Ca^{2+} oscillations measured in a single *E. viridis* cell in the presence of 1 mM $SrCl_2$ in the external medium. Ca_{cy} was measured at a sampling frequency of 1/3 s^{-1} after mechanical microinjection of the fluorescent Ca^{2+}-sensitive dye fura-2-dextran. **b** Sr^{2+}-induced Ca^{2+} oscillation calculated by numerical integration of Eq. (14.7), using the following parameter set: $\nu_{in}=1$, $k_{out}=1$, $k_{ero}=2$, $k_{erL}=0.05$, $k_{eri}=1.2$, $K=2.5$, $n=4$, $Sr_{cy}=0.1$, $\rho_{er}=1$, and with $Ca_{cy}(t=0)=0.2$ and $Ca_{er}(t=0)=24$ as start values. **c** Calculated values of Ca_{cy} are plotted against calculated values for Ca_{er}, displaying the so-called limit cycle. Starting at $Ca_{cy}(t=0)=0.2$ and $Ca_{er}(t=0)=24$, the two variables quickly approach the limit cycle. For sake of simplicity, no units are given and the axes are given in arbitrary units for the calculated Ca^{2+} oscillation (in **b** and **c**; see text)

and Ca_{cy}-axes in nM, additional parameters would have to be introduced – such as the Ca^{2+} buffer capacity – and reasonable values for most parameters would have to be available. However, for most of the rate constants, affinities, volumes, buffer capacities, etc. needed for a more detailed model, no established values exist. For the time being, therefore, more detailed models with additional parameters are more likely to introduce additional uncertainty than to achieve higher accuracy. To solve the mathematical functions developed here (Eq. 14.7), most parameters were given values close to 1. The activity of the ER Ca^{2+}-ATPase (k_{ero}=2) is assumed to be twice as high as the activity of the plasma membrane ATPase (k_{out}=1), and the leak releasing Ca^{2+} from the ER (k_{erL}=0.05) is assumed to have a much lower rate constant than that for the Sr^{2+}/Ca^{2+}-induced Ca^{2+} release (k_{eri}=1.2) – all these seem to be reasonable assumptions.

In Fig. 14.4c, Ca_{cy} is plotted against Ca_{er}. The closed curve in this plot is referred to as limit cycle. From their start values, the two variables ($Ca_{cy}(t=0)$=0.2, $Ca_{er}(t=0)$=24) quickly approach this limit cycle and then cycle in a fixed orbit. For stable oscillations, the size and shape of the limit cycle depend on the parameter set used but not on the start values of the two variables. Regardless of the start values, the oscillation will always culminate in exactly the same limit cycle (Stucki and Somogyi 1994). Stable oscillations are therefore also referred to as limit cycle oscillations.

Starting with the parameter set used in Fig. 14.4, one can now change the values for different parameters and observe how this affects the calculated oscillations. During the investigation of Sr^{2+}-induced Ca^{2+} oscillations in *E. viridis*, it had been observed that an increase in external Sr^{2+} concentrations resulted in faster oscillations with smaller amplitudes (Bauer et al. 1998a); analogous observations were made when different amounts of Sr^{2+} were injected directly into the algal cell. Changing the value for Sr_{cy} in Eq. (14.7) results in a similar behaviour for the calculated oscillations. Keeping all other parameters as in Fig. 14.4, no oscillations are observed below Sr_{cy}=0.08. Increasing Sr_{cy} results in an increasing frequency, from a wavelength of λ=64 at Sr_{cy}=0.08 to λ=14.3 at Sr_{cy}=0.54. Amplitudes start decreasing already at Sr_{cy}>0.11 and, at about Sr_{cy}=0.5, amplitudes become very small, resulting in 'saw tooth' shape oscillations. At Sr_{cy}=0.55, no oscillation occurs. The dose dependence of frequency and amplitude of Sr^{2+}-induced Ca^{2+} oscillations recorded in *E. viridis* can qualitatively be reproduced by the mathematical model described by Eq. (14.7).

This good correspondence between experimental data and the mathematical model does not, of course, prove that the model is correct. Nonetheless, it demonstrates that even a highly simplified model is sufficient to reflect the Sr^{2+} dose dependence observed. The assumption of a constant Sr_{cy} itself is also a simplification and, in a more realistic model, the Sr^{2+} concentration in the cytoplasm (Sr_{cy}) and in the ER (Sr_{er}) could be treated as variables. Ultimately, this would result in a 'doubling' of the equations and parameters used in Eq. (14.7). As discussed above, however, this would add quite a bit of complexity without necessarily providing new insight.

Which range of values for a single parameter does result in oscillations depends on the values of the other parameters. Keeping all other parameters as in Fig. 14.4, no oscillations are observed at v_{in}=0.2 (rather than v_{in}=1). However, there are oscillations at v_{in}=0.2 if k_{eri} is increased to 15.7 *and* K is decreased to 1; neither an increase of k_{eri} to 15.7 alone or a decrease of K to 1 alone is sufficient for oscillations. Obviously, it is impossible to predict which parameter values will result in oscillation and which will not. With a software package such as Mathematica©, it is easy to solve Eq. (14.7) numerically for a variety of parameter sets but, for a more systematic study, a stability analysis should be performed. This is done by calculating the so-called eigenvalues for different parameter sets, with positive eigenvalues indicating stable oscillations. For an excellent introduction of the concept of a stability analysis, the reader is referred to Stucki and Somogyi (1994).

For a more informal approach, there are some basic requirements for stable oscillations – as mentioned above – that have to be fulfilled. A system with at least two variables is needed for simple oscillations; three or more are required for bursting or chaotic Ca_{cy} dynamics (Schuster et al. 2002). At least one feedback connection between the oscillating variables as well as 'sufficient total nonlinearity' are required (Stucki and Somogyi 1994). Frequently, this nonlinearity results partly from the nonlinear feedback between the variables; typical elements here are cooperative binding or Michaelis-Menten-type binding. Obviously, for a Ca^{2+} oscillator – a system that is able to produce stable Ca^{2+} oscillations – it needs more than only a Ca^{2+} pump and a Ca^{2+} channel (Harper 2001).

Could the mathematical model of Ca^{2+} oscillations described here for the unicellular green alga *E. viridis* be used to describe Ca^{2+} oscillations in other plant cells? Most likely, yes – with some modifications, of course. In a pharmacological study of Nod factor-induced Ca^{2+} oscillations in root hairs of *Medicago truncatula* (Engstrom et al. 2002), the ER Ca^{2+}-ATPase blockers DBHQ or CPA effectively inhibited Ca^{2+} oscillations at µmolar concentrations – as in *E. viridis*. Caffeine and 2-APB (2-aminoethoxydiphenylborate), modulators of ryanodine receptor and/or IP_3 receptor Ca^{2+} channels, inhibited Nod factor-induced Ca^{2+} oscillations, too (Engstrom et al. 2002). This indicates that, comparable to the model presented here, the second variable might be Ca_{er} or the Ca^{2+} concentration of the nuclear envelope, since Nod factor-induced Ca^{2+} oscillations were shown to initiate in the nuclear region of root hair cells (Ehrhardt et al. 1996). On the other hand, externally applied La^{3+} or Gd^{3+} (1 mM) had no apparent effects on Nod factor-induced Ca^{2+} oscillations (Engstrom et al. 2002), indicating that a model with constant total cellular Ca^{2+} content (v_{in}=0, v_{out}=0) might be more appropriate.

The most attractive system for modelling Ca^{2+} oscillations in plants seems to be stomatal guard cells. Guard cells are the most extensively studied system dealing with the generation of Ca^{2+} oscillations in plant cells (see Chapters 7 & 8). A variety of stimuli – including ABA, external Ca^{2+}, cold and H_2O_2 – can induce Ca^{2+} oscillations in guard cells, and there is good evidence

that different molecular mechanisms are involved in Ca^{2+} oscillations induced by different stimuli (Allen et al. 2000; Evans et al. 2001; Hetherington and Brownlee 2004). Mathematical modelling should help to clarify the different pathways that are available in guard cells to generate Ca^{2+} oscillations.

Moreover, Ca^{2+} oscillations in guard cells show different shapes and complexities. In *Commelina communis* guard cells (McAinsh et al. 1995), 0.1 mM external Ca^{2+} (Ca_{ext}) induces simple, regular Ca^{2+} oscillations (called symmetrical oscillations by McAinsh et al.) whereas 1.0 mM Ca_{ext} induces bursting oscillations (called asymmetrical oscillations by McAinsh et al.). These issues are described in detail by McAinsh in Chapter 7 of this book. 'Bursting' describes a pattern in which phases of high-frequency oscillations are separated by phases of quiescence and, in Ca^{2+} bursting, usually each phase of oscillation – or each burst – consists of an initial large spike followed by a series of smaller spikes. Simple oscillations, also called regular spiking, can be modelled by relatively simple models with two variables and are often based on CICR – like the model developed above (Eq. 14.7). Bursting oscillations, whether regular or chaotic, require more complex mathematical models with at least three variables and three differential equations (Borghans et al. 1997; Schuster et al. 2002). Mathematical modelling of the transition from simple to bursting Ca^{2+} oscillations at increasing Ca_{ext} in *C. communis* guard cells might give new insight into the molecular mechanisms generating those Ca^{2+} oscillations.

Ca^{2+} influx across the plasma membrane (v_{in} in Fig. 14.1) has been shown to be involved in the generation of Ca^{2+} oscillations in guard cells of different species and under different stimuli (McAinsh et al. 1995; Klüsener et al. 2002). Hyperpolarization-activated plasma membrane Ca^{2+} channels (Hamilton et al. 2000; Pei et al. 2000) seem to play a key role in Ca^{2+} influx into guard cells. This indicates that, in a mathematical model of guard cell Ca^{2+} oscillations, the membrane potential has to be introduced as a separate variable. In experiments with *Vicia faba* guard cells, oscillations in the potential of the guard cell plasma membrane were accompanied by Ca_{cy} transients (Grabov and Blatt 1998). Artificial hyperpolarization of the guard cell plasma membrane results in an increase of Ca_{cy} (Grabov and Blatt 1998; Allen et al. 2000). Accordingly, a mathematical model for regular Ca^{2+} oscillations in guard cells may be based on two variables – Ca_{cy} and the plasma membrane potential. What is the evidence for feedback between these two variables? The membrane potential regulates Ca^{2+} influx in a nonlinear manner. Increasing Ca_{ext} seems to stimulate the opening of hyperpolarization-activated plasma membrane Ca^{2+} channels whereas increasing Ca_{cy} reduces their activity (Hamilton et al. 2000). The Ca^{2+} influx (v_{in}) affects the membrane potential directly whereas increasing Ca_{cy} affects the membrane potential indirectly by regulating different plasma membrane ion channels. In a mathematical model of guard cell Ca^{2+} oscillations, hyperpolarization-activated Ca^{2+} channels would be a central element. They connect the two variables – Ca_{cy} and membrane potential – and, in addition to being regulated by Ca_{cy} and Ca_{ext}, they seem to

be the target of a multitude of signalling pathways that are involved in stomatal closure (Klüsener et al. 2002; Hetherington and Brownlee 2004).

In addition to Ca^{2+} influx across the plasma membrane, there are good indications that Ca^{2+} release from intracellular stores contributes to the generation of Ca^{2+} oscillations in guard cells. A phospholipase C blocker inhibits ABA-induced Ca^{2+} oscillations (Staxen et al. 1999; Klüsener et al. 2002), indicating the importance of intracellular Ca^{2+} release via IP_3 receptor Ca^{2+} channels. Although there is no experimental evidence indicating which intracellular Ca^{2+} store might carry these IP_3 receptors, it seems justified to speculate that oscillations in the Ca^{2+} concentration of a Ca^{2+} store (such as Ca_{er} in Eq. 14.7) can be involved in guard cell Ca^{2+} oscillations – as in many other systems. With three oscillating variables, Ca_{cy}, Ca_{er} (or another store) and the membrane potential, different models for regular Ca^{2+} oscillations can be developed, and even bursting Ca^{2+} oscillations in guard cells might eventually be described by a plausible mathematical model.

Ca^{2+} oscillations in plant cells are a fascinating but not very well-understood phenomenon. One reason for this paucity of information is the complex nature of oscillations. There is good evidence that, as in animal cells, Ca^{2+} oscillations in plant cells are involved in intracellular signal transduction processes, and that the frequency and amplitude of these oscillations might encode the information to be transmitted (Ng and McAinsh 2003; Hetherington and Brownlee 2004). This means that different stimuli cause Ca^{2+} oscillations of different frequencies and amplitudes and, depending on frequency and amplitude, different physiological reactions are triggered, enabling stimulus-specific reactions. Yet, the encoding mechanisms setting frequency and amplitude remain elusive. There is good experimental evidence on which molecular components might be involved in the generation of intracellular Ca^{2+} oscillations. However, how these components bring about an oscillation with a certain frequency and amplitude is not understood, and can probably not be without mathematical modelling.

The best way to make reasonable predictions about the frequency and amplitude of a Ca^{2+} oscillation is on the basis of a mathematical model. Theoretical predictions based on mathematical modelling can then be tested experimentally to refine the mathematical model and to improve our understanding of the physiological functions of Ca^{2+} oscillations.

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15 Noise-Induced Phenomena and Complex Rhythms: Theoretical Considerations, Modelling and Experimental Evidence

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Abstract

Combining the unravelling of the molecular bases of functions in time and of organization in space in biology, on the one hand, with nonlinear dynamics as part of theoretical physics, on the other, is promising great progress in basic understanding of nonlinear spatial pattern formation from huge amounts of data becoming available in systems biology. In this chapter, this will be assessed in terms of the "tripod" (1) experimentation, (2) modelling and (3) theory.

(1) As empirical case studies we will mainly use in this chapter spatiotemporal dynamics of crassulacean acid metabolism and stomatal pore regulation by guard cells. These can be documented by, among other techniques, chlorophyll fluorescence imaging. This leads to modelling and theoretical treatment of circadian and ultradian endogenous oscillations. (2) In modelling, maximum models, providing perfectionist "photographic" imaging of nature, are distinguished from minimal models singling out essential domains in the parameter space of systems, with heuristic aims. The latter are explored in approaches based on experiment/theory feedback. (3) Theoretical assessment dwells on the method of cellular automata, which are frameworks for simulating spatiotemporal patterns arising from local interactions. The theoretical concepts developed are based on the examination of stochasticity with the order-generating effects of noise in stochastic resonance and coherence resonance, where intermediate noise intensity generates quasi-rhythmic behaviour of systems from arrythmicity.

This merges into a new path towards systems biology, where the huge amount of data currently provided by analytical progress is cast into the concept of universal dynamic principles. We illustrate this new path by using simple models of synchronization, this being one concept which systems biology can then exploit for the construction of advanced models.

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Rhythms in Plants: Phenomenology, Mechanisms, and Adaptive Significance

15.1 Introduction

Among the most conspicuous scientific success stories of the last few decades are, on the one hand, the unravelling of the molecular bases of functions in time and of organization in space in biology and, on the other, the study of nonlinear dynamics as part of theoretical physics. Completely new dynamical phenomena have first been derived from theoretical constructs and then been demonstrated experimentally in nature. Most prominent among these ideas is the functional role of noise, in particular stochastic resonance as a new view towards optimization (Gammaitoni et al. 1998; Moss 2000), and self-organized criticality resulting from long-term evolution as a unifying thought in ecosystem dynamics (Bak et al. 1988; Solé et al. 1996).

In this vein, existing empirical observations of organization over time in space (e.g. in developmental biology) or over a space in time (e.g. in biorhythms) strongly profit from subsequent theoretical analysis of the underlying mechanisms of self-organization. Indeed, both aspects of fundamental research are needed because, as Alfred Gierer writes (Gierer 1998), to understand the system properties of the living world, one needs experimental observations and data, an appropriate abstract perception of the experienced reality and – not the least – suitable mathematical concepts. However, even though many branches of the life sciences have quickly grasped the intriguing ideas of nonlinear dynamics and have by now exploited these for more than 20 years, in some fields the use of such methods is still regarded as a somewhat exotic and, certainly, non-standard way of looking at a system. This may be due to the nontrivial mathematical tools involved. Unfortunately, plant biology is among those fields, perhaps also because it has particularly strongly integrated the molecular point of view. To return to developmental pattern formation as one of the most outstanding examples – and quoting Alfred Gierer again – we may note that, notwithstanding the great achievements of molecular biology, not all explanations are necessarily exclusively and primarily molecular; systems of components have properties which the components themselves do not have, and to grasp these properties is the task of nonlinear system theories. The most interesting processes in the living world, and among them, developmental pattern formation, can be understood only with a combination of mathematical and system-theoretical approaches (Gierer 1998).

On these grounds, we need to ask which experimental observations could be subjected to an extended analysis, using theoretical approaches. Beforehand, however, we have to know about these approaches. Apart from extending knowledge in the realm of nonlinear dynamics, the theoretical research efforts of the last few years have also made these approaches much more applicable. In many areas, promising theoretical results have helped starting joint projects between biology and physics. Here, we illustrate one way in which this rather new technology of nonlinear dynamics, if applied, may lead to an important breakthrough in plant biology.

We will describe some of the general concepts and explicit methods which serve as tools for the formulation of mathematical models for biology and for the analysis of experimental data. The first focus of this chapter is on the mathematical description of plant metabolism, which helps understand oscillatory phenomena in plants. Under certain conditions, such systems can produce spatial patterns, which change with time. Spatiotemporal dynamics is, therefore, the second key topic of our chapter. Our third centre of interest is a concept which has implications for both the nonlinearities of the systems at hand and the capacity of the systems to produce patterns: *noise*. In all these cases, we will show how abstract theoretical concepts are linked with explicit biological observations, and how the notion of noise-induced behaviour can help understand biological systems.

The vivid dialogue between theory and experiment is essential to these examples. As plant physiologists, we will rarely, if ever, be able to actually apply the theoretical methods and, as theorists, we cannot design and execute the experimental approaches – evidently, mutual learning of a communal language is vital. In the following, this will be identified as the most difficult but most important ingredient of nonlinear dynamics applications to biology, which will always remain an interdisciplinary endeavour.

15.2 Case Study I – Crassulacean Acid Metabolism (CAM)

Most plant biologists are familiar with modelling. Modelling is performed for metabolic reaction sequences (Mulquiney and Kuchel 1999; Niinemets et al. 1999; Poolman et al. 2000) and cycles (Blasius et al. 1998). Growth/respiration relations have been modelled by Taylor et al. (1998). Due to the fundamental importance of vegetation for climate, most global-change models in some way incorporate plants and plant physiology, in particular in relation to CO_2 budgets (e.g. Medlyn et al. 1999). Photosynthesis models addressing various functions of photosynthesis, including stomatal regulations, have found a very wide application (e.g. Niinemets and Tenhunen 1997; Haefner et al. 1997; Pearcy et al. 1997; Walcroft et al. 1997). For the understanding of natural and agronomic ecosystems, modelling is performed at higher scaling levels of canopies (e.g. De Pury and Farquhar 1997; Magnani et al. 1998), forests (Williams et al. 1998; Medlyn et al. 1999), ecosystems (Running 1990) and globally (Woodward 1987). Remote sensing for the supply of photosynthesis-related data is becoming increasingly sophisticated, involving measurements of laser-induced chlorophyll fluorescence transients. A photochemical reflectance index enables assessment of light use efficiency based on the dynamics of the xanthophyll cycle, which serves for non-photochemical energy dissipation under stress. Thus, the spatiotemporal heterogeneity of the outer surface of forest canopies can be depicted and this will also include observations from space (for review, see Schurr et al. 2006).

What are the major objectives behind modelling? We consider there are three important aims: (1) to depict reality, (2) to understand reality and (3) to make predictions. The former two need models of opposing nature with respect to their significance for experimental work:

1. The development of a *maximum model* as close to reality as possible, providing a perfectionist "photographic" image of nature and enabling fine-tuned simulations, which can be regarded as a true alternative to experiments,
2. the design of a *minimal model*, which accounts only for the most important properties of the system and attempts to enhance the experiment's efficiency by singling out domains in the parameter space of the system, where knowledge of the system's dynamical behaviour is essential for the understanding of the system itself.

An interesting account of the aims of maximum models is given, e.g. in Kohl et al. (2000). Conversely, in this review we focus mostly on minimal models. A minimal model (or skeleton model) is a mathematical model description which approximates a biological system by taking into account only the *relevant* mechanisms for an observed dynamical behaviour. The question is then no longer *How close does one get to the dynamics of the original biological system?* but rather *What ingredients are really necessary for the model to reproduce the essential traits of the system's dynamics?*

A standard form of mathematical model is given by ordinary differential equations.[1] In such equations, the change of a dynamic variable with time is again a function of the variable itself. Properties of the system are then expressed in terms of the functional link between the variable (i.e. the state of the system at time t) and the temporal change (i.e. the time derivative of the dynamic variable). The general form of such a (one-dimensional) ordinary differential equation is given by

$$\frac{\mathrm{d}x}{\mathrm{d}t} = f(x) \tag{15.1}$$

Given an initial value $x(0)$, the function yields the change $\mathrm{d}x/\mathrm{d}t$ from this point on, leading to a new value of x a little later and, thus, to a new function

[1]Although ordinary differential equations are a frequent approach to modelling, a wide variety of other descriptions exists. One passes to partial differential equations when, in addition to changes in time, the spatial behaviour is taken into account. When a focus is on the influence of fluctuations on the dynamics, stochastic differential equations are analyzed. Often, formulations which are discrete in space or time are selected due to their smaller computational demands and the capacity to incorporate local rules, which are not easily cast into the form of differential equations. Finite-difference equations for the purely temporal case and cellular automata in the case of spatiotemporal patterns are examples of such formulations. We will briefly discuss cellular automata within the context of stomatal dynamics.

value $f(x)$ which, in turn, determines a new change dx/dt. In this way, the differential equation, Eq. (15.1), *encodes* the function $x(t)$. This function $x(t)$ is the solution of the differential equation. One can couple such simple cases of (one-dimensional) differential equations to form more complex models. In all such cases, the terms on the right-hand side of such equations can be interpreted as specific sources of change of the system's dynamic variables.

In order to illustrate these ideas, we will describe an explicit case study, namely the cycle of CAM and its circadian endogenous oscillations. CAM is a modification of C_3-photosynthesis in which, under normal ambient conditions, CO_2 is fixed nocturnally via phospho*enol*pyruvate carboxylase (PEPC). This leads to the formation of malic acid (or its anion malate), which is transported across the tonoplast membrane for storage in the central cell sap vacuole, remobilized during the following daylight period, decarboxylated, and the CO_2 regained assimilated in the light via ribulose-bis-phosphate carboxylase/oxygenase (RubisCO), as in normal C_3-photosynthesis. CAM provides an excellent model system for the interdisciplinary study of circadian rhythmicity, as it displays a variety of dynamical phenomena which are of interest from the viewpoint of nonlinear dynamics. The most important dynamical event is the switching from vacuolar malate loading to vacuolar malate remobilization.

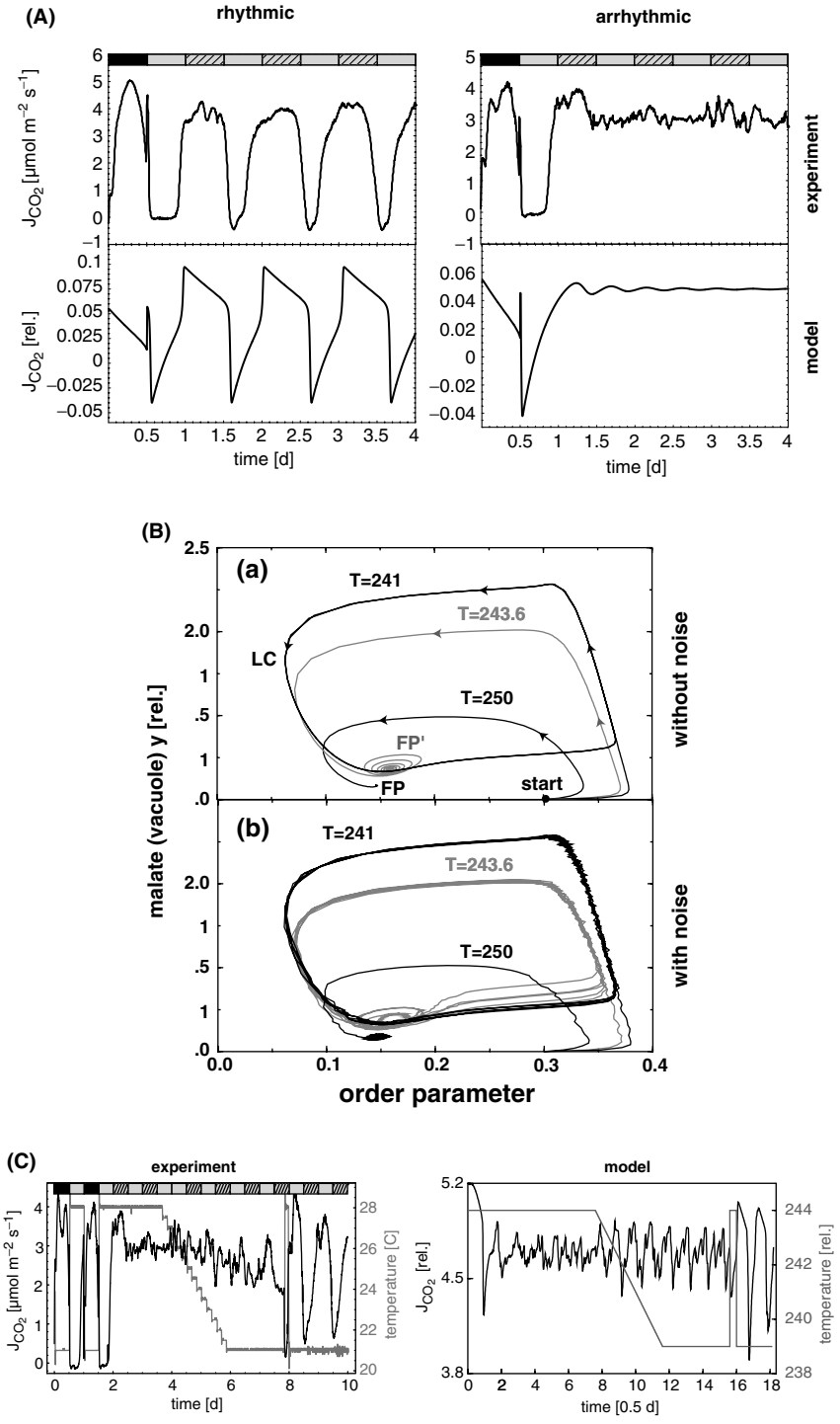
In the diurnal cycle under normal conditions, a conventional explanation of this switching would be feasible on the basis of equilibrium thermodynamics: the equilibrium of nocturnal malate loading is disturbed by fundamental changes of external conditions with the onset of the light period, due to irradiance and associated change of temperature, so that the switching to malate unloading has a ready explanation – as does, vice versa, the switching to loading again. In addition to light, temperature is an important control parameter in the regulation of these dynamics, as it affects the fluidity of the tonoplast lipo-protein membrane and, hence, malate permeability (Kluge et al. 1991; Kliemchen et al. 1993; Lüttge 2000). However, the fact that CAM is also free running as an endogenous rhythm with circadian oscillations of CO_2-exchange (J_{CO2}) and malate levels, due to switches between vacuolar loading and unloading in continuous light and under strictly constant conditions, clearly points to the nonlinear dynamics of CAM.

Well-known central clock genes of higher plants, such as *TOC1* (*timing of CAB – chlorophyll a,b binding protein – expression*), *CCA1* (*circadian clock associated*) and *LHY* (*late elongated hypocotyl*), are rhythmically expressed in CAM plants and, most interestingly, in a species, *Mesembryanthemum crystallinum* L., which can switch from C_3-photosynthesis to CAM, the phases of these oscillating genes are offset against each other in both modes of photosynthesis (Boxall et al. 2005). Moreover, a gene encoding PEPC-kinase, which modulates PEPC activity, a key function in CAM, is known to be a clock-controlled gene (CCG). However, the transcription of this apparent key gene is under metabolic control of malate, and its biophysical compartmentation between cytoplasm and vacuole, i.e. dependent on vacuolar loading/unloading via the tonoplast membrane (Borland et al. 1999; Lüttge 2000; Nimmo 2000).

Thus, although there is evidence of a *TOC1/CCA1/LHY*-oscillator to control oscillations in the CAM pathway (Hartwell 2005), CAM is also an example where functioning of a biophysical circadian oscillator downstream of the direct involvement of genetic control can be demonstrated, and where a direct involvement of clock-controlled gene(s) appears to be excluded and the primary oscillator of the CAM cycle is based on posttranslational functions.

Therefore, application of nonlinear dynamics tools was an important choice for advancing our understanding of the CAM oscillator. Over one and a half decades, a close collaboration has been established between plant physiologists and theoretical physicists, resulting in the development of dynamical minimal models (Lüttge and Beck 1992; Blasius et al. 1997, 1999; Lüttge 2000) as well as in several general contributions to nonlinear dynamics (e.g. Hütt and Neff 2001; Busch et al. 2001). Studies with the CAM plant *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie have revealed that, above a certain threshold temperature, J_{CO2} changes from rhythmic to arrhythmic behaviour and that this is reversible when temperature is lowered again (Lüttge and Beck 1992). It has been shown that these findings are well reproduced by minimal models of six, four (for which mathematical details are summarized in, e.g. Lüttge 2000) or even only three coupled nonlinear differential equations with temperature, light intensity and external CO_2 concentration as external parameters (Fig. 15.1a).

Fig. 15.1 Typical simulation results obtained with the CAM models summarized in Lüttge (2000). For comparison, the corresponding experimental findings are also shown. **a** The elementary model consisting of six metabolite pools (as described in the text) schematically reproduces the rhythmic (below the upper temperature threshold) and the arrhythmic (above the upper temperature threshold) gas exchange patterns. **b** The same behaviour is displayed for the refined model without noise (*a*) and with noise (*b*), illustrating the dynamical properties of the model more clearly. A phase diagram is shown which gives the relation between two of the dynamical variables, namely the vacuolar malate content *y* and the lipid order parameter of the tonoplast. In such a phase diagram, time is a parameter along the curves (trajectories), as indicated by the *arrows*. In *a*, the trajectories for three different temperatures are given. One sees a limit cycle (LC) at the lowest temperature and a rapid convergence into a fixed point (FP) at the highest temperature. At an intermediate value of temperature, the system slowly spirals into the fixed point which is now slightly shifted (to FP′), as the equilibrium states themselves depend on temperature. When noise is included in the theoretical description (*b*), the behaviour of the former two trajectories remains qualitatively the same. The intermediate case, however, displays a completely different form of dynamics, as now the spiralling process is interrupted frequently by stochastic kicks onto the nearby limit cycle, which the system leaves after a full oscillation to again approach the fixed point. The advanced version of the model is now capable of producing realistic time series even for rather complex experimental protocols. An example of this is given in **c**. Here, a slow change from a temperature associated with arrhythmic behaviour to a temperature in the rhythmic regime (i.e. weak dynamics of signal input) is applied with the astonishing effect of observing an arrhythmic gas exchange pattern even in a temperature domain of usually rhythmic behaviour, which is restored after a sudden temperature pulse (i.e. strong dynamics of signal input). Due to the new ingredient of noise-induced desynchronization, the model is capable of reproducing this phenomenon. Model parameters (as well as the temperature in **b**) are shown in relative units (adapted from Hütt and Lüttge 2002)


(A)
rhythmic
arrhythmic
experiment
model
time [d]
(B)
(a)
(b)
T=241
T=243.6
T=250
LC
FP'
FP
start
without noise
with noise
malate (vacuole) y [rel.]
order parameter
(C)
experiment
model
temperature [C]
temperature [rel.]
time [d]
time [0.5 d]

Due to the combination of a highly controllable experimental setup and a consistent theoretical representation, we may regard the endogenous circadian CAM rhythm of *K. daigremontiana* as a very suitable model system for the study of the biological clock. We show here how the vivid exchange of concepts and ideas between model and experiment can lead to a completely new view of the functioning of this biological rhythm: the biological clock as a spatiotemporal phenomenon. One crucial tool in our interpretation of CAM dynamics is a mathematical model (Lüttge and Beck 1992; Blasius et al. 1997) which has been formulated on physiological grounds. The dynamics of six metabolite pools (malate in the cytoplasm, malate in the vacuole, internal CO_2-concentration, glucose-6-phosphate, phospho*enol*pyrovate (PEP) and starch) are determined by fluxes between these pools, which depend on three external control parameters (light, temperature, external CO_2-concentration). A wide variety of experimental results for the CAM plant could be reproduced in the past by this model and refinements thereof. Examples are shown in Fig. 15.1. Some of the refinements are worth mentioning, as they have immediate implications for a physiological understanding of CAM dynamics and, in addition, illustrate the idea of a *minimal* model:

1. Mathematical considerations regarding the different time constants of the processes involved enabled the elimination of three of the metabolite pools in the glycolytic pathway, namely starch, glucose-6-phosphate and PEP, showing that observations can be explained by the interacting dynamics of internal CO_2 and cytoplasmic and vacuolar malate alone (cf. Blasius et al. 1999 for details).
2. The thermodynamical properties of the tonoplast membrane during CAM have been analyzed independently (Neff et al. 1998). It could be shown that, in the model, the average lipid area density serves as a control parameter, with lipid order undergoing a phase transition when this parameter is varied. The lipid order parameter then has the role of a fourth dynamical variable in the three-pool model. The model is capable of accounting for the existence of a rhythmic and an arrhythmic domain, where the former is represented in terms of a limit cycle whereas the latter is described by a fixed point of the system of differential equations (cf. Fig. 15.1).

At this stage, the model can be thought of as a single circadian oscillator which, depending on external conditions, is either in a stationary state (fixed point) or in an oscillatory state (limit cycle). However, the idea of a single oscillator is quite contrary to the picture one gets experimentally. Here, one may expect the leaf to consist of different domains or patches which, under certain conditions, may behave as independent oscillators – in other words, each leaf cell performing CAM may contain an autonomous copy of the oscillator (Rascher et al. 2001). For the theoretical description, this means that one has to consider an ensemble of identical, independent or weakly coupled oscillators, rather than a single oscillator.

3. The next refinement is closely related to this major step from a purely temporal to a spatiotemporal dynamics. It concerns the inclusion of stochastic processes and dynamics at a small time scale, which formally enter the description as *noise*.

In the last years, it has been appreciated that unveiling the effect of noise in a (nonlinear) system can substantially contribute to the understanding of the processes within the system. Noise is a ubiquitous phenomenon and, in most cases, a disturbance to the measuring process. From the viewpoint of nonlinear dynamics, however, it may very well influence a system's dynamics in a more sophisticated way than only by overshadowing it. It is possible that some biological process (e.g. the detection of a signal) functions in an optimal way only at some intermediate noise level present in the system (Douglass et al. 1993; Bezrukov and Vodyanoy 1995; Gammaitoni et al. 1998; Anishchenko et al. 1999; Moss 2000). With lower or higher noise intensity, the efficiency of the process will decrease. A schematic example of such a *stochastic resonance* is given in Fig. 15.2a. This phenomenon is possible only in a nonlinear system (in our schematic example of Fig. 15.2a, the threshold serves as nonlinearity). This is by now theoretically well understood and has been observed, together with the related effect of coherence resonance (Pikovsky and Kurths 1997), in a variety of natural and model systems (Longtin et al. 1991; Moss et al. 1994; Bezrukov and Vodyanoy 1995; Lee et al. 1998; Beck et al. 2001).

Let us return to the CAM model. As soon as noise is included in the theoretical description, the arrhythmic gas exchange pattern is reproduced realistically and more complex scenarii, such as a slow (rather than abrupt) temperature variation, are simulated by the model, yielding high agreement with experimental data (Fig. 15.1c).

Two important new features enter the system due to the presence of noise. First, it is possible for the oscillators to loose synchronization: when several identical noisy CAM oscillators are used in the simulation (with the net CO_2 exchange being the average over this ensemble), and if no external *zeitgeber* is present, then the oscillators will desynchronize as time evolves. Second, spontaneous, noise-induced transitions from the fixed point to the limit cycle become possible (Fig. 15.1b). Indeed, these transitions display the highest correlation with the internal frequency of the CAM oscillator at intermediate noise strength. This phenomenon, named coherence resonance (Pikovsky and Kurths 1997), is similar to the stochastic resonance mentioned above (see also Fig. 15.2b).

Triggered by the theoretical suggestion of a loss of synchronization of distinct leaf areas, the experimental study of CAM dynamics has been advanced to the point where spatial and temporal variations over a given leaf can be investigated using the non-invasive technique of photographic chlorophyll fluorescence imaging to depict the synchronization and desynchronization of photosynthetic activity in leaf patches (Rascher et al. 2001; Rascher and

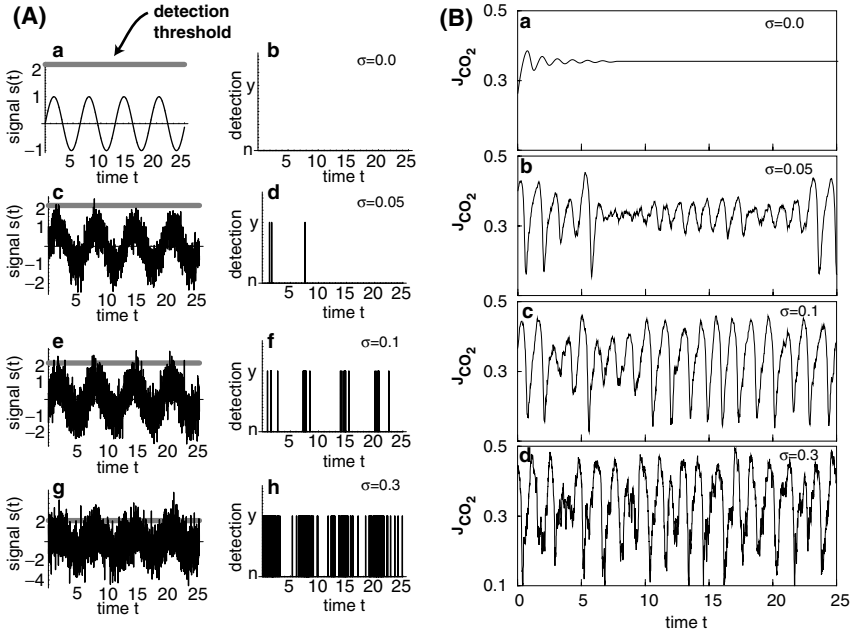


Fig. 15.2 a Schematic example of a system displaying stochastic resonance. A signal $s(t)$, consisting of a sine wave and additive noise, enters some detection mechanism with a threshold (*a*, *c*, *e* and *g* for different noise intensities σ). At each point in time, the signal is translated into yes (y) or no (n), depending on whether the signal reaches above the threshold or not (*b*, *d*, *f* and h). One sees that the sequence of maxima present in the pure signal (i.e. without noise, as seen in *a*) is best reproduced by this device at an intermediate noise intensity. In a natural system, the more frequent case is that a (almost) pure signal enters a system with a noisy threshold. Mathematically, these two cases are equivalent. **b** The CAM model, which is illustrated in Fig. 15.1, shows a similar behaviour with respect to recovering the circadian oscillation in the noisy fixed point. In *a* is a schematic representation of the system without noise (σ=0) reaching the fixed point. Curves in *b*, *c* and *d* were obtained by numerical simulation with increasing noise intensities (σ of 0.05 to 0.3). At a medium noise intensity (*c*), the circadian rhythm is optimally recovered (coherence resonance). At higher (*d*) and lower (*b*) noise intensity, the CO_2 gas exchange curve J_{CO2} shows a much more irregular pattern (adapted from Hütt and Lüttge 2002)

Lüttge 2002). Internal CO_2 and its lateral diffusion within the leaf airspaces have been identified as an essential signalling element for synchronization (Duarte et al. 2005; Lüttge and Hütt 2006).

At the level of data analysis, graph variants of neighbourhood-based spatiotemporal analysis tools (see Hütt and Neff 2001 and Hütt and Lüttge 2002) have been formulated and extended to a method for estimating connectivity from the time series of oscillators on a graph (Hütt and Lüttge 2005a). Additionally, the impact of graph theory and network dynamics on plant physiology has been reviewed from a similar perspective (Hütt and Lüttge 2005b).

15.3 Case Study II – Stomatal Patterns

Stomatal patterns in leaves are determined by a variety of external and internal control parameters interacting in signalling networks. This makes up the complexity of stomatal rhythms (see Sect. 15.4 below). The ultimate pacemaker of guard cell movements for stomatal opening and closing, however, appears to be CO_2 (Raschke 1965; Lüttge and Hütt 2006). Although a CO_2 sensor and receptor molecule has not been definitely identified as yet, it is clear that increased and decreased internal CO_2 concentrations effect stomatal closing and opening respectively. Spatiotemporal patterns of stomatal opening/closing states are then dependent on the homobaric or heterobaric nature of leaves (Neger 1918; Terashima 1992). Homobaric leaves have no conspicuous anatomical constraints for internal CO_2 diffusion, so that internal partial pressures of CO_2 are homogenous (homobaric) and, consequently, stomatal states are also largely homogenous over the leaves. Heterobaric leaves have anatomical vein arrangements hindering gas diffusion, which leads to the well-known spatiotemporal phenomenon of stomatal patchiness (Beyschlag and Eckstein 1997). Under particular conditions in the absence of the typical anatomical features of heterobaric leaves, the leaves may become physiologically heterobaric due to physiological constraints (Duarte et al. 2005; Lüttge and Hütt 2006).

In spite of its intrinsic lack of quantitative comparison with a precise mathematical model, from our point of view the study by Peak et al. (2004) constitutes a huge progress in conceptually understanding the spatiotemporal behaviour of stomatal guard cells. By observing that stomata basically have to compute an opening state optimal at the global (leaf-wide) level using only local (cell–cell) communication, the authors compare stomatal dynamics with a model system from complexity theory, namely cellular automata (CA).

Let us look at this type of modelling in more detail. A cellular automaton is a framework for simulating spatiotemporal patterns arising from local interactions. Figure 15.3 summarizes this concept. In the case of one spatial dimension (1D CA), one has a chain of elements, each of which can be in a particular state out of a state space Σ. Update rules translate neighbourhood constellations at time t into the state of the central element at the next time step t+1 (see Fig. 15.3a). By consecutively applying the update rules, one can simulate the time evolution of such a chain of elements corresponding to this particular selection of update rules. A strength of cellular automata lies in the simplicity of both the concept and the state space of a specific model, which usually contains only few distinct states. Consequently, the idea of cellular automata is not to describe a biological phenomenon as accurately as possible but rather to grasp the essence of a system in terms of few degrees of freedom. In this sense, cellular automata represent an extreme case of minimal model for biological situations.

As this brief summary of cellular automata already shows, this model operates in a discrete space, at a discrete time, and in a finite and discrete

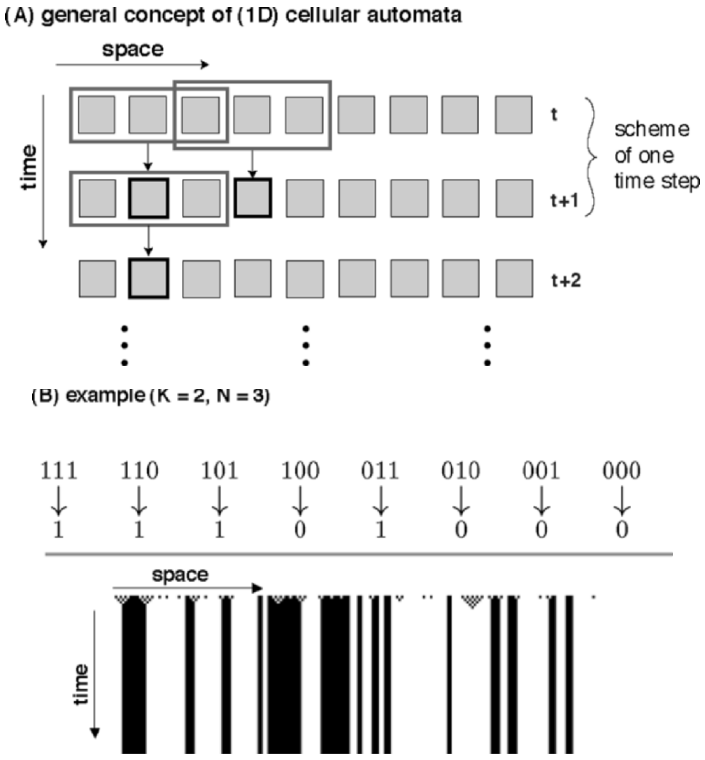


Fig. 15.3 Concept of cellular automata (CA) illustrated for one spatial dimension (**a**) and an example of a CA simulation for a binary state space and a three-element neighbourhood (**b**). For this selection of update rules, one obtains fixed-point dynamics after a short transient (adapted from Lüttge and Hütt 2006)

state space. Figure 15.3b gives an example of a simple cellular automaton. This example considers three-element neighbourhoods (N=3) and a binary (two-element) state space (K=2), e.g. containing only 0 and 1. In this case, 2^3=8 neighbourhood constellations have to be specified. The example given in Fig. 15.3b leads to a steady-state pattern, after a short transient.

This general framework of cellular automata has received much scientific attention as a laboratory for studying complex systems (see, e.g. Wolfram 2002). Moreover, specific cellular automata have proven useful as minimal models for aspects of biological pattern formation ranging from simplest Turing patterns to the characteristic spiral wave patterns observed in excitable media. Even against this wide background, however, it is not readily understood how this CA-type of model could mimic the behaviour of stomatal dynamics. The interesting approach by Peak et al. (2004) has been to analyze the statistical features of a rather famous cellular automaton designed to perform a specific computational task, and compare these features with the computational task they record in stomatal spatiotemporal dynamics.

The cellular automaton they consider is a so-called *density classifier*. In its simplest form, the computational task of density classification starts from a random distribution of 0*s* and 1*s*, and then uses local rules to determine the state with the highest global density. Note that, with the help of an external agent, this would be a trivial computational task requiring only to count, e.g. the number of 1*s* in the initial state. With no external agent and using only local interactions between the elements, the computation of the state with the highest initial density is far from trivial. This situation has been studied extensively in complexity theory (see, e.g. Mitchell et al. 1994; Crutchfield and Mitchell 1995; Wolfram 2002). A simple example of such a density classification is the *majority rule*, where the update rule maps an element to the state which holds the majority in the neighbourhood under consideration. In order to advance one step closer to the biological phenomenon, we consider the case of two spatial dimensions. Figure 15.4 summarizes the update rule, which retains some features of the majority rule with the additional condition of shuffling undecided neighbourhoods. Here, the update rule operates on the number of 1*s* in the nine-element neighbourhood for this automaton in two spatial dimensions. Update rules involving only the number of, e.g. 1*s*, rather than their distribution in the neighbourhood, are called *totalistic cellular automata* in CA theory. Figure 15.5 shows spatial snapshots at

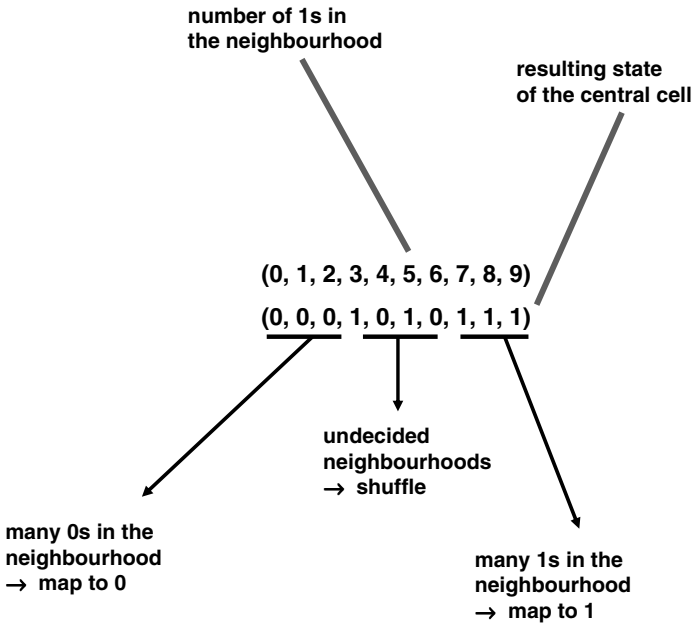


Fig. 15.4 Summary of the update rule for a totalistic CA suitable for density classification. The *upper line* of digits in parentheses gives the number of 1*s* in a spatial (nine-element) neighbourhood whereas the *lower line* lists the corresponding state of the central cell in the next time step (adapted from Lüttge and Hütt 2006)

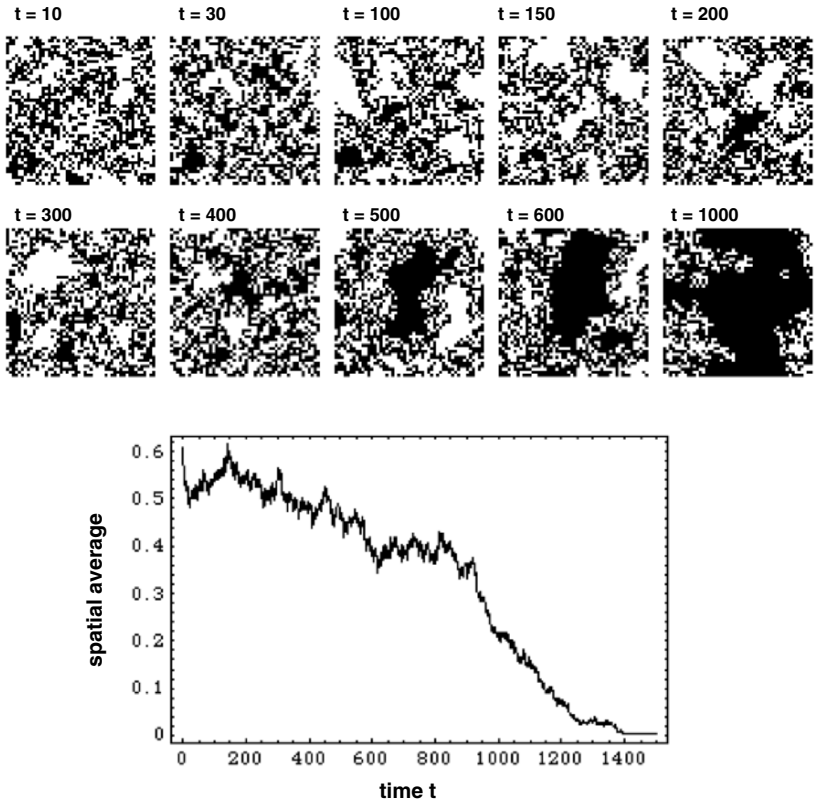


Fig. 15.5 Spatial snapshots of the CA in Fig. 15.4 at different time points (*top*) and time course of the density $r(t)$ of 1*s* (i.e. of the spatial average over the lattice at each time point; adapted from Lüttge and Hütt 2006)

different time points starting from an initial density of 1*s*, p_1, close to 0.5. The corresponding time course of the density is displayed in the bottom part of Fig. 15.5. The capacity of this automaton to classify initial densities by converging to a specific state is summarized in Fig. 15.6. Here, densities $\rho(t)$ for different initial densities, p_1, are compared. It is seen that whereas highly biased densities are rapidly classified by this automaton, the difficult cases, where $p_1 \approx 0.5$, can lead to long transients (and also to an occasional misclassification). Peak et al. (2004) evaluate such cellular automata in terms of the event size distributions and other statistical parameters, and compare these quantities with the stomatal patterns. Both in theory and experiment, they focus on the long transients.

This approach is conceptually different from the usual framework of theoretical biology, where specific mathematical models are formulated and then analyzed with methods of nonlinear dynamics. Here, the mathematical

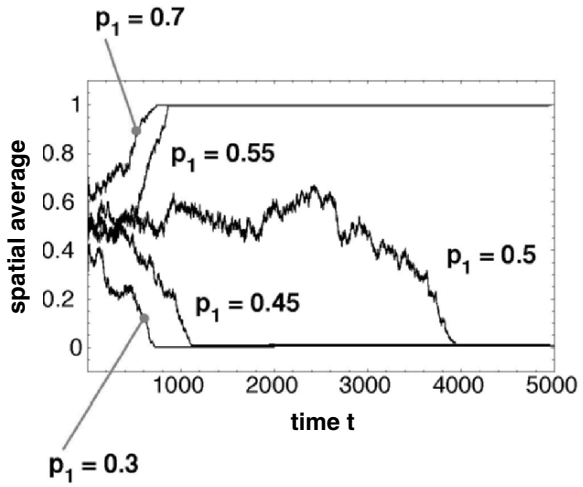


Fig. 15.6 Density $r(t)$ as a function of time t for different values of the initial density of 1*s*, p_1. The cellular automaton is the same as in Figs. 15.4 and 15.5 (adapted from Lüttge and Hütt 2006)

description focuses on analogies. A quantitative analysis then relies on comparing statistical properties of the real-life data with those from the theoretical counterpart. For stomatal dynamics, Peak et al. (2004) analyze event sizes, the scaling of transients, and the distribution of inter-peak intervals in the average opening state (see also West et al. 2005). One can view this study as an example of a new trend in theoretical biology on its way to an understanding at a system-wide level: ever larger systems and, on the other hand, an increasing importance of stochastic contributions (usually coming from few-molecule configurations beyond the usual average concentrations and far beyond the molecular level) require models with abstract, discrete dynamics and the use of stochastic processes to implement the effect of stochasticity at this abstract level (see also Bornholdt 2005).

15.4 Experimental Observations of Complex Rhythms in Plants

What are the origins of the high complexity of biorhythms? First, there are a variety of external control parameters, which may interact among each other. Second, the external control parameters feed in their signals via receptors into complex input networks with primary and secondary messengers. Third, the oscillators themselves are networks composed of a hierarchy of clock elements constituting the knots of molecular, biochemical, biophysical and physiological components interconnected by the edges of the networks.

Fourth, the output pathways again are networks of feedback-regulated reaction systems, i.e. subsystems of the output machinery. Fifth, even the overt output oscillations may feed back information into the entire system (Lüttge 2003b; Lüttge and Hütt 2004). This forcefully underlines the strong nonlinearity as well as the entry of noise as a factor establishing ordered responses in biorhythmic phenomena, which can be grasped only by approaches incorporating all three legs of the magic tripod, viz. experimentation, theory and modelling – where our metaphor by no means implies witchcraft!

Biorhythm research is currently dominated by the quest for the biological clock or circadian oscillator on a genetic basis (for a review of the plant biological literature, see Lüttge 2003a). Some central oscillator genes or master genes have been identified (e.g. see Sect. 15.2 above). Although we must keep in mind that, in *Drosophila*, both the circadian rhythm of eclosion and locomoter activity and the very short ultradian rhythm of the male courtship song seem to be governed by the same PER gene (Dunlap 1993), it is highly debatable whether there is one central gene oscillator at the very top of the hierarchy of biological clock systems. The complexity must be larger, and major current progress at the molecular level clearly shows that we are dealing with genetic networks and multiple oscillator systems, rather than with one central oscillator (Lüttge 2003a). Nevertheless, the search for a dominant molecular oscillator has somewhat possessed present research.

Conversely, in studies of ultradian rhythmicity, more emphasis appears to be given to the consideration of biochemical, biophysical and physiological elements and the required theoretical treatments of empirical data, and much can be learnt from the study of high-frequency rhythms (for review, see Lüttge and Hütt 2004). Spontaneous ultradian-frequency rhythms remain a central topic in neurobiology with the electrical firing of neurons, and oscillations of cyclic adenosine-monophosphate (cAMP) secretion, the attraction and pattern formation of amoebae of the slime mould *Dictyostelium* (e.g. Berridge and Rapp 1979), and the oscillatory behaviour of glycolysis (e.g. Berridge and Rapp 1979; Olsen and Degn 1985; Rapp 1986) are much studied. However, astonishingly little research effort is devoted to ultradian high-frequency oscillations in plant biology. This is regrettable because, in this case, it is often much easier to keep the systems studied under strict experimental control and, also, sufficiently extended time series of measurements for theoretical analysis can be obtained more readily than is the case for the much longer circadian oscillations. Moreover, studying high-frequency oscillations generally also implies that we set ourselves free from being "paralyzed" by the evolutionary and genetic biological clock enigma. Among the ultradian cellular oscillators, Berridge and Rapp (1979) distinguish two types: (1) cytoplasmic oscillators, in which the periodic phenomena are generated by an instability in metabolic pathways and (2) membrane oscillators, generating rhythms of membrane potential. In the large variety of ultradian rhythms known, there is no evidence for a common oscillatory mechanism at the cellular level (Berridge and Rapp 1979). Therefore, in some contrast to circadian rhythms, we appear to be

becoming much more open to system analysis to unravel governing principles of nonlinear dynamics. This is generic, and it may be one of the reasons for the high success of studies of, e.g. *Dictyostelium* and glycolysis. This has much bearing also on our understanding of circadian rhythmicity (see Lüttge 2003a).

The two examples from plant biology on which we predominantly dwell in this chapter, stomata and CAM, are chosen because they clearly illustrate the five ingredients of complexity listed above and because they are comparatively well studied.

Ever since the discovery of stomata as elements of an oscillatory CO_2 regulation system (Raschke 1965), stomata have been a key example of cybernetics in plants. Ultradian oscillations of stomatal aperture and conductivity for water vapour have been reported over decades (Barrs 1971; Teoh and Palmer 1971; Cowan 1972a, b; Kaiser and Kappen 2001; Roelfsema and Hedrich 2002). Primary external signals or control parameters are blue and red light, hydraulic effects and leaf internal airspace CO_2 (Willmer 1988), affecting a highly complex internal regulation network. Cytoplasmic Ca^{2+} levels play a key role as a central node in this network (McAinsh et al. 1997), and a plasma membrane Ca^{2+} channel may be an important oscillator in the system (McAinsh et al. 1995). Interrelated subsystems involved are stomatal guard cell movements as such and photosynthesis. Due to the intimate interaction of photosynthetic metabolism and gas exchange, it is difficult and often impossible to distinguish between oscillations of stomata and photosynthesis. Oscillations of stomata and photosynthetic output parameters begin after abrupt changes in external parameters such as light, CO_2, O_2, H_2O and temperature (Walker 1992; Giersch 1994). However, there are also spontaneous stomatal oscillations (Raschke 1975). In a seminal review, Giersch (1994) considers the various possible metabolic control points and discusses two different mathematical minimal or "skeleton" models simulating oscillations. One model is based on a "two-kinase hypothesis" (see also Giersch et al. 1991), with phosphoglycerate and phosphoribulose kinases, and the other is based on a "coupling hypothesis", with the interdependence of photosynthetic electron flow and photophosphorylation. Evidence is in favour of the former hypothesis.

In CAM, the major interrelated subsystems are (1) malate compartmentation, (2) stomatal movements and (3) CO_2-assimilation via Rubisco (Bohn et al. 2003). The overwhelming complexity here is illustrated by recent observations that the vacuolar malate accumulation/remobilization oscillator discussed in Sect. 15.2 above, i.e. subsystem 1, appears to be only part of the whole story. In *K. daigremontiana*, endogenous circadian malate oscillations are highly dampened within three to four circadian periods, without any dampening of the overt output rhythm of net CO_2-exchange (Wyka and Lüttge 2003; Wyka et al. 2004). This implies that a delicate, fine-tuned internal regulation of oscillator and output networks must lead to takeover of subsystems 2 and/or 3, without any disturbance of the output itself and perhaps under the control of a genetic *TOC1/CCA1/LHY*-oscillator (see Sect. 15.2).

15.5 A Path Towards Systems Biology

By adding to the standard approaches of theoretical biology, particularly the construction of minimal models, biology is currently at the threshold of addressing functional issues by means of integrative approaches at a system-wide level. Systems biology is emerging from genome sequencing. It first aims at collecting data on cells ("cellome") and organisms (Raikhel and Coruzzi 2003) with "accurate measurements of thousands of molecules from complex biological samples" (Sweetlove et al. 2003). The conceptual idea behind this is comprehensive modelling, i.e. "the complete mathematical description of a model plant species" (Sweetlove et al. 2003) – in our view, this would be called a maximum model (Sect. 15.2). However, then a key ingredient in the transition to systems biology becomes integration of data on the spatial and temporal organization of biological units at very different scales. Beyond the existence of a particular gene in the genome, such a *systems biology* approach draws attention to several questions – under which external conditions is this gene expressed, which other genes regulate its expression, which external control parameters (e.g. temperature and resources) characterize the external conditions, and many other aspects.

Our understanding of circadian rhythms is a good example of such a transition towards an integrative approach. In the cyanobacterium *Synechococcus elongatus*, almost all genes and, in the angiosperm *Arabidopsis thaliana* (L.) Heynh., very many genes are clock controlled (CCGs; Liu et al. 1995; Michael and McClung 2003).

The process of integrating system-wide information from several scales is the core competence of systems biology. Within a slightly narrower definition based on (computational) systems biology, one understands the step from laboratory experiments (either with living organisms, in vivo; or with subsystems – e.g. cell cultures, in vitro) to computer simulations (here, the term in silico seems now widely accepted) in order to understand system properties. In this approach, one assumes that a (mathematical) model representation of the system exists which is close to reality. Therefore, such systems biology approaches deviate from the minimal model concept, which attempts to grasp the essential systemic ingredients (for a particular set of phenomena; e.g. rhythmicity) with the smallest mathematical effort (or, more precisely, with the least complex model).

The task of systems biology – exploiting all empirical data to yield in silico simulations of the system – is tremendously difficult. On the one hand, this comes from the complexity inherent in real biological systems. On the other hand, this task is complicated enormously by the sheer complexity of even very simple mathematical description. From our point of view, the general aims of systems biology can, therefore, only be accomplished by exploiting universal dynamic principles. These may provide a framework into which system-biology approaches can be incorporated. Indeed, to a certain extent they represent, in a very condensed form, a wealth of information on dynamic

systems. In order to appreciate both the complexity of a systems biology approach as well as the potential patterning of realistic descriptions by universal dynamic principles, we will briefly discuss the minimal modelling of rhythmic phenomena as well as an example of such a universal dynamic principle relevant to rhythmicity, namely the emergence of spontaneous synchronization at an increasing coupling of dynamic elements.

This impressive example of a universal dynamic principle which may provide fundamental guidelines for a path towards systems biology has been discovered by Winfree (1967), and it has been cast into a minimal model by Kuramoto (1984). As a collective dynamic phenomenon, synchronization has many facets in nature. Fireflies synchronize their flashing, leading to the dramatic visual perception of rhythmically flickering trees, and crickets synchronize their sound, just as concert visitors their applause. In epilepsy, a synchronous firing of many neurons is an essential part of the pathological dynamic state. Many forms of spatiotemporal pattern formation in biology require a (spatially organized) synchronization of functional units, e.g. to establish spiral waves and other forms of propagating patterns.

Discussing synchronization/desynchronization, we may distinguish between qualitatively different types of rhythmic output, which may be due to qualitatively different underlying oscillators, and qualitatively identical rhythmic output based on multiple copies of qualitatively the same oscillator. There are many examples of different types of rhythms expressed even within the same cells, and in both prokaryotic and eukaryotic unicellular organisms, as reviewed recently by Lüttge (2003a). Mostly, these rhythms are not synchronized. Synchronization of multiple copies of functionally identical units is an important problem for theoretical analysis of nonlinear dynamics of spatiotemporal pattern formation. In plant biology, examples of time scales in synchronization/desynchronization range from rather short periods to those as long as, e.g. 30–40 years for the flowering of bamboo plants. For the shorter time scales, again we may return to stomata. Synchronization/desynchronization, of course, is involved in stomatal non-patchiness/patchiness (see Sect. 15.3 above). Individual oscillators or leaf patches also play a role in whether or not overall gas exchange of a leaf is seen to oscillate. Cardon et al. (1994) have demonstrated patchy distribution of stomatal oscillations in sunflower using chlorophyll fluorescence imaging and gas exchange techniques. Siebke and Weis (1995a, b) also used chlorophyll fluorescence imaging. With the assimilation images obtained, they observed that rapid changes in gas composition initiated oscillations in net gas exchange (H_2O and CO_2) which changed to non-synchronous oscillations due to slight local variations in the period. Obviously, oscillations in net gas exchange died out but assimilation persisted oscillating non-homogenously and non-synchronously over areas, patches or spots of the leaves (Siebke and Weis 1995a). Gas diffusion within the leaf may play a role in this respect (see Sect. 15.3; Cardon et al. 1994; Siebke and Weis 1995a) but minor-vein distribution and its mediation of transport processes (e.g. sugar export) also appears to be involved (Siebke and Weis 1995b).

Still, how unavoidable is synchronization? How does it depend on external influences on the system? Do forms of partial synchronization exist, where only a subset of dynamic elements is synchronized? If so, which properties qualify an element to be part of the synchronous group? In order to understand the phenomenon of synchronization, we will look at it from a more formal perspective. Figure 15.7 shows the "generator" of our periodic signal, which will be the focus of our discussion. A point moves with uniform (angular) velocity on a circle of radius r in a plane. The position of the point can best be described in polar coordinates (ϕ, r). In terms of differential equations, one can summarize the dynamics simply by

$$\frac{\mathrm{d}\phi}{\mathrm{d}t} = \text{const} = \omega \tag{15.2}$$

This simple system is called a phase oscillator. Here, the parameter ω is the eigenfrequency of this oscillator. A highly nontrivial level is reached when several such units are coupled. A frequent form of coupling is given by the sine of phase differences. For two such oscillators with eigenfrequencies ω_1 and ω_2, one has:

$$\frac{\mathrm{d}\phi_1}{\mathrm{d}t} = \omega_1 + \varepsilon \sin(\phi_2 - \phi_1),\ \frac{\mathrm{d}\phi_2}{\mathrm{d}t} = \omega_2 + \varepsilon \sin(\phi_1 - \phi_2) \tag{15.3}$$

The parameter ε is the coupling strength. One can now study the synchronization of the two oscillators as a function of ε and of the two eigenfrequencies ω_1 and ω_2. Figure 15.8 shows the phase *difference* $\Delta\phi = \phi_1 - \phi_2$ at fixed

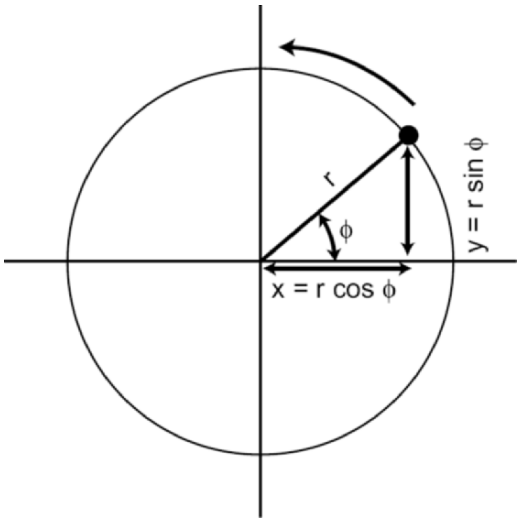


Fig. 15.7 Concept of a phase oscillator: schematic representation of a uniformly rotating point on a circle, together with the position given in Cartesian coordinates (x, y) and polar coordinates (r, ϕ; adapted from Hütt and Dehnert 2006)

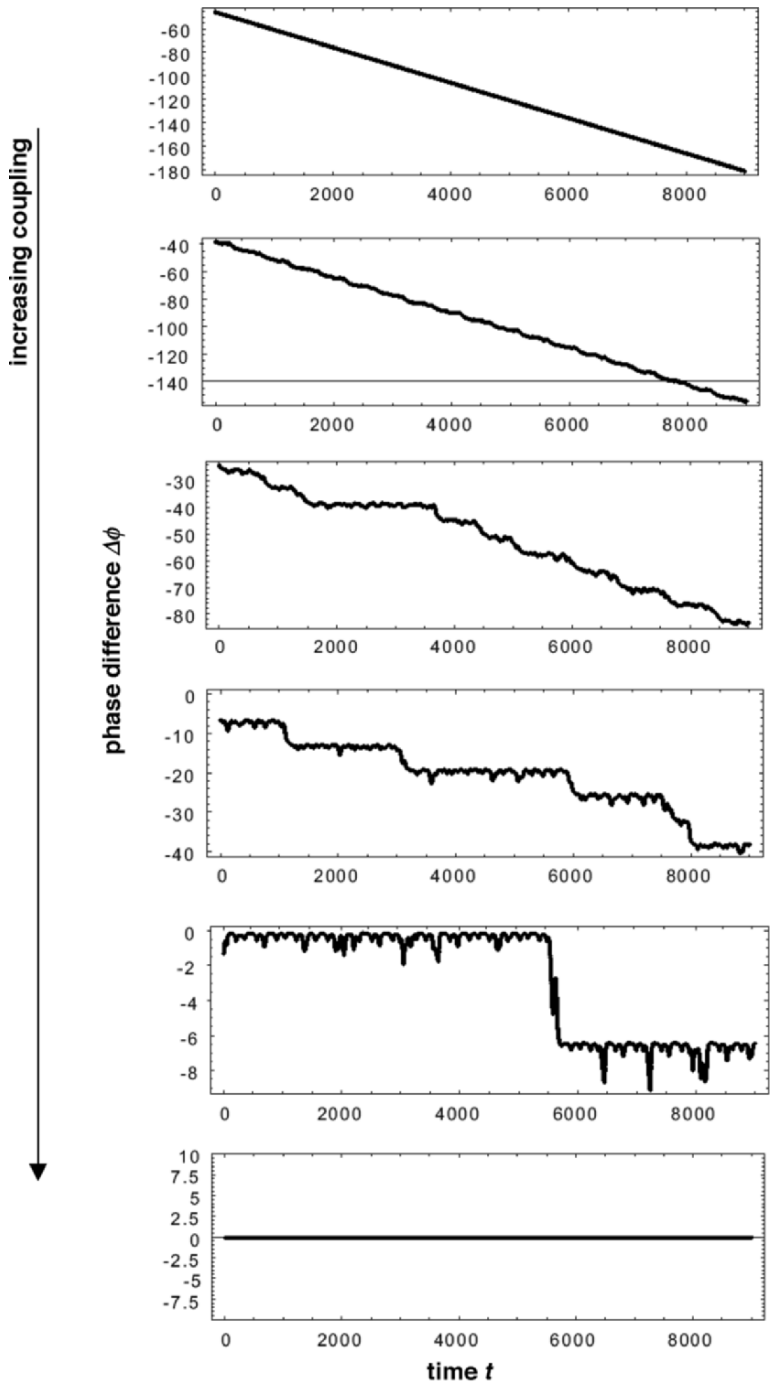


Fig. 15.8 Phase difference $\Delta\phi$ as a function of time t at different values of the coupling strength ε for a system of two coupled phase oscillators. The eigenfrequencies are ω_1=−0.7 and ω_2=−0.4. Coupling strength ε has been increased from ε=0 (*top*) to ε=1.0 (*bottom*) in steps of 0.2 (adapted from Hütt and Dehnert 2006)

eigenfrequencies for increasing coupling. One can see that, at low coupling, the phases are independent, leading to a continuous change in $\Delta\phi$. At higher coupling, a significant cross-talk between the two phases appears. Over rather long time intervals, the phase difference is constant. For two oscillators with different eigenfrequencies to have a constant phase difference over time, they must adjust their respective frequencies to a common frequency (i.e. they must "synchronize"). Beyond Fig. 15.8, one can visualize this process by analyzing the *effective* frequencies (i.e. the frequencies the coupled oscillators actually have) as a function of coupling strength. These effective frequencies Ω_1 and Ω_2 are given by the average phase change in a certain time interval. Figure 15.9a shows these effective frequencies Ω_1 and Ω_2 as a function of ε. At zero coupling, these effective frequencies coincide with the eigenfrequencies ω_1 and ω_2 from Eq. (15.3), as should be the case. At higher coupling, one observes the gradual adjustment of the two frequencies towards each other and, ultimately, the onset of synchronization.

In a similar form, we can now discuss a larger set of phase oscillators. In Fig. 15.9b, the effective frequencies for a group of ten such oscillators are

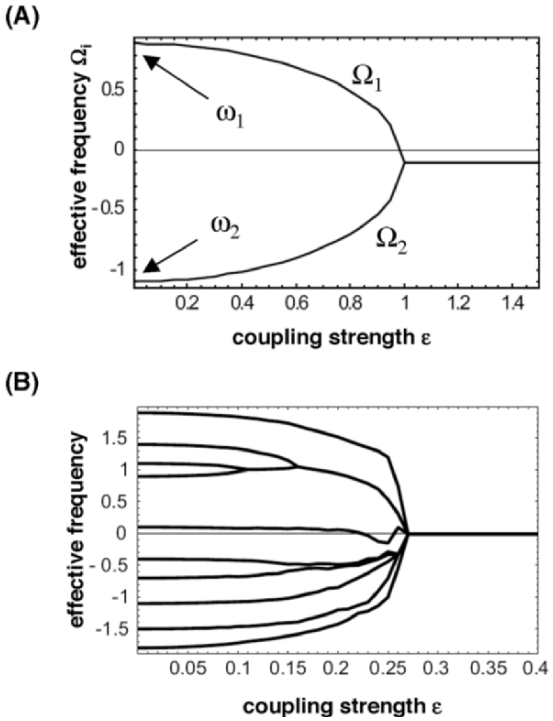


Fig. 15.9 a Effective frequencies Ω_1 and Ω_2 as a function of the coupling strength ε for the system of two coupled phase oscillators from Fig. 15.8. **b** Same as **a** but for ten coupled phase oscillators (adapted from Hütt and Dehnert 2006)

shown as a function of the coupling strength ε. A route towards complete synchronization paved by a step-wise synchronization of oscillators with similar eigenfrequencies is clearly seen. At a certain critical value of ε, all oscillators synchronize. This spontaneous onset of synchronization, when passing a critical coupling, is a minimal model of virtually any synchronization phenomenon in nature. At this point, the concept of universal dynamic principles discussed above becomes clear: a wide variety of individual phenomena is explained by a single principle. Furthermore, by having a universal description for the basic phenomenon of synchronization, it is now possible to identify additional effects in the individual systems beyond the basic phenomenon. An excellent non-mathematical introduction to synchronization is the book by Strogatz (2004). Systems biology can exploit such universal dynamic principles as backbones for the construction of more detailed and sophisticated models.

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16 Modeling Oscillations of Membrane Potential Difference

Mary Jane Beilby

Abstract

The oscillation of the membrane potential difference (PD) is considered in terms of one or more ion transporters changing their conductances. For slow oscillations (period greater than about one minute), the transporters could be identified by employing the current–voltage (I/V) technique. The electrical characteristics of each transporter population were then modeled and the evolution of the model parameters with time obtained.

Examples of single transporter type changing against a steady background conductance were the proton pump in the charophyte *Chara australis* and a putative potassium pump in *Ventricaria ventricosa*. A detailed modeling was done for the former, but it is in early development for the latter. The hypertonic regulation in the salt-tolerant charophyte *Lamprothamnium* spp., and an interaction of the proton pump and the proton channel in *C. australis* are given as examples of two transporters responding to a change in salinity and membrane PD, respectively. A detailed modeling was done for each response. The hypotonic regulation in *Lamprothamnium* involves a complex interaction of several transporters, initiated by the decrease of the medium salinity. Finally, spontaneous action potentials (APs) in *C. australis* are shown as the medium salinity is increased.

The examples demonstrate the analytical and predictive power of the I/V methodology coupled with the systems biology modeling.

16.1 Introduction

Oscillations of the membrane potential difference (PD) imply two or more PD equilibrium points to which the membrane PD can be attracted. The oscillation can be set up by changes in a single membrane transporter, which produces PD oscillations against some steady background PD and conductance

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level. In more complex situations, several transporters cooperate through PD-dependent gating, Ca^{2+} activation or mechano-activation.

From an experimental viewpoint, the measurement of PD changes alone is not sufficient for modeling of the oscillating system. If the frequency of the oscillation is slow, then data logging of the current–voltage (*I*/*V*) characteristics of the cell provides sufficient information to identify current contribution of each transporter and their evolution with time (Beilby 1990; Beilby and Walker 1996; Beilby and Shepherd 2001a, b, 2006a). The *I*/*V* characteristics can be obtained in 8 s (Beilby 1990). For oscillations with periods shorter than a minute, simultaneous conductance and PD measurements are needed to distinguish the transporter activation and inactivation.

In a living experimental system, it is not possible to physically disconnect different types of transporters to measure their contribution to the whole cell electrical characteristics. The use of channel blockers and metabolic inhibitors is often fraught with artifacts. Fortunately, the artifacts can be recognized and the total currents deconstructed into contributions by various types of transporters by integrative modeling, systems biology. Although there is a great multitude of transporters, only a few dominate the membrane conductance at any one time. Consequently, comparatively simple models can approximate the cell electrical characteristics under a range of conditions, with analytical and predictive power. This chapter illustrates such an approach to several plant electrophysiological responses. The data were gathered from giant celled plants: (1) charophytes, which are the ancestors of land plants (Beilby and Shepherd 2006b) and make an excellent model for these, and (2) *Ventricaria* (*Valonia*) spp., which represents siphonous green algae with different survival strategies in a marine environment (Bisson et al. 2006). Some of the models, such as that of the hypotonic regulation, are well advanced and have already provided useful analysis and feedback for experimental design. Others, such as the electrical characteristics of the K^+ pump in *Ventricaria*, or the spontaneous action potentials in salt-stressed *Chara* spp., are still in their initial stages.

16.2 Single Transporter Oscillations

16.2.1 Proton Pump and the Background State in Charophytes

The outer membrane of plant cells tends to be at more negative PD than that of animal cells due to the action of the proton pump (H^+ ATPase). Membrane PDs as negative as −350 mV have been recorded in *Chara corallina* (Lucas 1982). The protons flow back into the cell down the resulting electrochemical gradient $\Delta\mu_H$, facilitating the import of nutrients into the cell. However, the pump is not always fully activated. In some charophytes, such as *Chara inflata*, the pump switch is diurnal (Coleman and Findlay 1985). There might

be also a seasonal switch, as *Chara australis* cells in the winter months sometimes exhibit less negative resting PDs (Beilby, unpublished data).

When the pump activity is low, the PD attractor is the "background state". This state exhibits resting PD (E_{bkg}) close to –100 mV and linear current–voltage (I_{bkg}/V) characteristics in the vicinity (±100 mV) of E_{bkg}. The identity of the I_{bkg} carriers is not clear at present, but there is abundant experimental evidence for this membrane state in both *Chara* spp. and *Lamprothamnium* spp. (Beilby 1984, 1985; Beilby and Shepherd 2001a, b). In *Lamprothamnium*, the background conductance G_{bkg} increases with ionic strength, while E_{bkg} remains close to –100 mV (Beilby and Shepherd 2001a). The G_{bkg} does not increase if the medium osmolarity is increased by adding sorbitol (Al Khazaaly and Beilby, unpublished data). Interestingly, if the ionic strength and osmolarity are stepped down, transient increase in G_{bkg} and depolarization of E_{bkg} can be observed (Beilby and Shepherd 2006a). We propose that at least some of the channels passing the I_{bkg} are mechano-sensitive. The E_{bkg} does not coincide with the Nernst potential of any ion abundant in the cell or the medium, suggesting that the channels are not very selective and may be the equivalents of NSCCs in higher plants (Maathuis and Sanders 2001; Tyerman 2002; Demidchik and Tester 2002). These channels could mediate the Na^+ and Ca^{2+} inflows, the former a main cause of salinity toxicity in plants, the latter important in regulatory signal cascades.

In our modeling (Fig. 16.1), I_{bkg} is represented by an empirical equation with PD-independent conductance, G_{bkg}, and reversal PD, E_{bkg}, near –100 mV:

$$I_{bkg} = G_{bkg}\,(V - E_{bkg}) \tag{16.1}$$

The dependency of pump current I_p on membrane PD (Fig. 16.1) has been fitted by the HGSS (Hansen, Gradmann, Slayman, Sanders) model (Hansen et al. 1981; Beilby 1984; Blatt et al. 1990 and references therein).

$$I_p = zFN\,\frac{k_{io}\kappa_{oi} - k_{oi}\kappa_{io}}{k_{io} + k_{oi} + \kappa_{io} + \kappa_{oi}} \tag{16.2}$$

$$k_{io} = k^0_{io}\,e^{\frac{zFV}{2RT}} \tag{16.2a}$$

$$k_{oi} = k^0_{oi}\,e^{-\frac{zFV}{2RT}} \tag{16.2b}$$

F, R, T are the Faraday constant, the Gas constant and temperature in degree Kelvin, z is the pump stoichiometry, which has been set to 1, N is a scaling factor (2×10^{-8}), and V is the PD across the membrane or membranes. The number of carrier states was reduced to two with a pair of PD-dependent constants, k_{io} and k_{oi}, with a symmetric Eyring barrier, and PD-independent rate constants, κ_{io} and κ_{oi}. The rate constants, κ_{io} and κ_{oi}, subsume ATP, ADP, inorganic phosphate concentrations, binding and de-binding steps, and carrier recycling (Blatt et al. 1990).

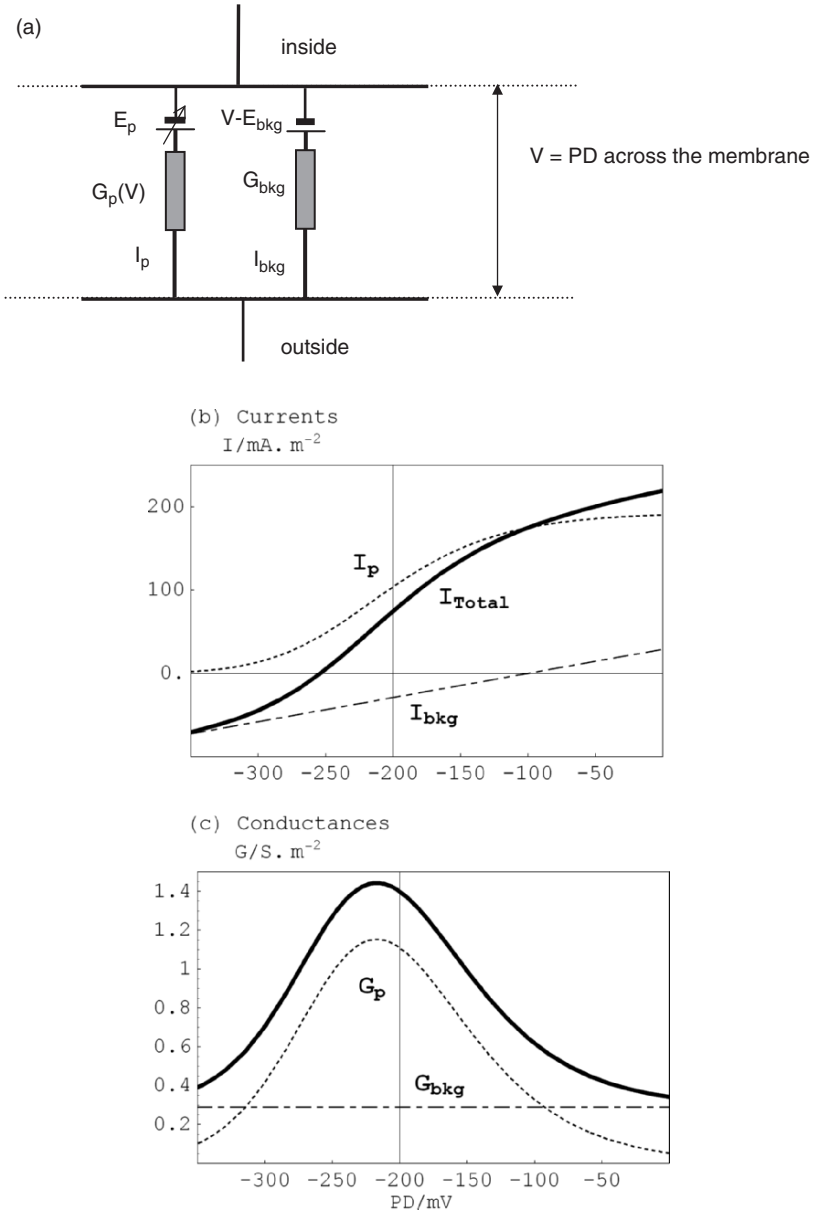

Fig. 16.1 a Simplified circuit of the membrane with only the background transporter and the pump. **b** Modeled total current (*continuous line*), I_p (*dotted line*) and I_{bkg} (*unequally dashed line*) as a function of the membrane PD. The parameters for the I_{bkg} are: G_{bkg}=0.29 S m^{-2}, E_{bkg}=−100 mV. The parameters for the I_p (in s^{-1}) are: k^0_{io} = 7,000, k^0_{oi} = 0.5, κ_{io} = 0.5, κ_{oi} = 100. **c** Conductances were obtained by differentiation of the currents (line types as in **b**)

The effect of the more influential parameters k_{io}^0 and κ_{oi} is explored in Fig. 16.2a and b, respectively (for all the parameter values, see the captions of Figs. 16.1 and 16.2). The decrease in both parameters causes the membrane PD to depolarize toward E_{bkg}, but the shape of the *I*/*V* characteristics is distinct in each case. The conductance–voltage (*G*/*V*) profiles, obtained by differentiation of the *I*/*V* profiles, display a clear maximum. For parameter k_{io}^0 decrease, the conductance maximum moves to more depolarized PDs, its amplitude decreasing only slightly (Fig. 16.2b), whereas diminishing κ_{oi} leaves the conductance maximum close to the same PD level, while its amplitude becomes smaller. The decrease in medium pH is a good example of the k_{io}^0 decrease (Beilby 1984); κ_{oi} decrease often manifests itself during a long experiment, perhaps due to diminished ATP in the cell. Mimura et al. (1983) found that the resting PD of perfused *Nitellopsis obtusa* cells depolarized below 400 μM ATP in open vacuole system, and 100 μM ATP in ligated cells. Unfortunately, the *I*/*V* characteristics were not measured. Smith and Walker (1981) found that increased ADP concentration in perfused *Chara* cells also depolarized the resting PD. The *I*/*V* curves were measured, but not over a sufficiently large PD interval to fit the HGSS model.

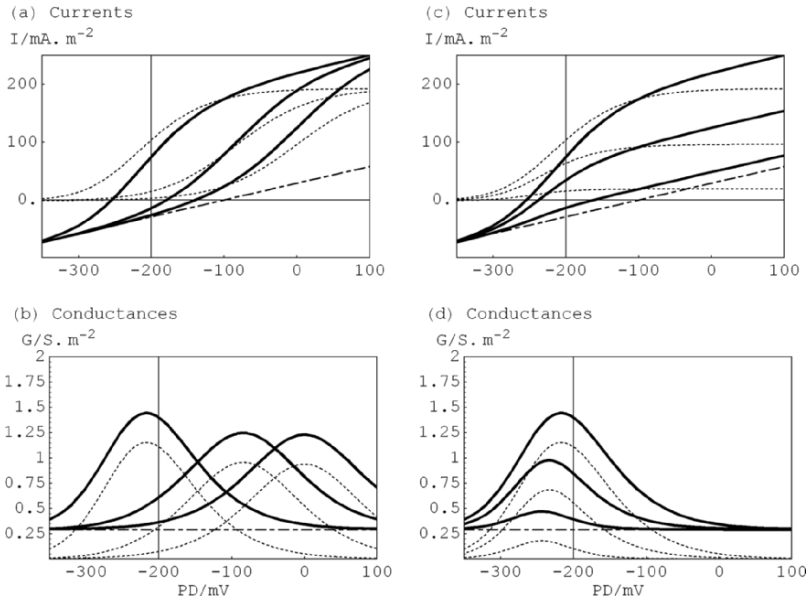

Fig. 16.2 Modeling changes in pump parameters that can produce depolarization. **a** Change in the k_{io}^0 parameter from 7,000 to 500 and to 100 s^{-1}, with I_{bkg}, the other pump parameters and the line types as in Fig. 16.1b. **b** Conductances calculated from the currents in **a**. Note the shift of the maximum toward more depolarized PD levels. **c** Change in the κ_{oi} parameter from 100 to 50 and to 10 s^{-1} (other parameters as in Fig. 16.1b). **d** Conductances calculated from the currents in **c**. Note that the conductance maximum remains at the same PD level, but its magnitude diminishes

16.2.2 Putative K^+ Pump and the Background State in *Ventricaria ventricosa*

Compared to charophytes and higher plants, the siphonous green algae have smaller membrane PD, which is positive under steady-state conditions. They are unlikely to have a proton-based chemiosmotic transport system. Rather, their membrane contains electrogenic K^+ ATPase (see Bisson et al. 2006 for review). We associate the high conductance at positive PDs (see *I*/*V* curve 2 in Fig. 16.3) with the activity of the K^+ pump (Beilby and Bisson 1999; Bisson and Beilby 2002). The background state has E_{bkg} near 0 mV and low G_{bkg} (*I*/*V* curve 1 in Fig. 16.3). The unusual electrophysiology stems from an uncommon cell structure with a sponge-like cytoplasmic layer, which can spontaneously divide into hundreds of viable aplanospores (see Shepherd et al. 2004 for review). The modeling of the pump and the background state is yet in early stages, but we are close to isolating the K^+ pump *I*/*V* characteristics. The cause for the pump oscillations at the time of impalement recovery is not known at present.

16.3 Two Transporter Interaction

16.3.1 Proton Pump and the Background State in Hypertonic Regulation in *Lamprothamnium* spp.

The salt-sensitive charophyte *Lamprothamnium succinctum* inhabits brackish lakes, where salinity fluctuates from close to freshwater after heavy rains up to twice seawater at times of drought. *Lamprothamnium* is able to regulate its turgor in this huge range of salinities (Bisson and Kirst 1980). As the medium becomes more saline, the water potential of the vacuole has to be lowered by importing more K^+, Cl^- and Na^+. This is an uphill process, and the energy comes from the activation of the proton pump. Greater salinity makes G_{bkg} more conductive (Beilby and Shepherd 2001a), so that the pump has to work even harder (see Fig. 16.4). Note that in this case, k^0_{oi}, k^0_{io} and κ_{oi} all increase (see the parameter values in Fig. 16.4 caption), so that the curve shape is a combination of those seen in Fig. 16.2. Note also that the changes in membrane PD are small, but the conductance near the resting PD is more than doubled. The *I*/*V* characteristics have been adequately modeled by the changes in the I_p and I_{bkg} in the PD range −100 to −275 mV. However, other transporters must be involved in this case. We suggest that K^+ is imported through the inward rectifier. In the data shown in Fig. 16.4, the inward rectifier was fitted with the GHK model (see Sect. 16.3.2), and there was a depolarization of the half-activation PD from −400 to −360 mV. However, monitoring of this current by clamping the membrane PD to more negative levels affects the proton pump (see next section), and the present results are

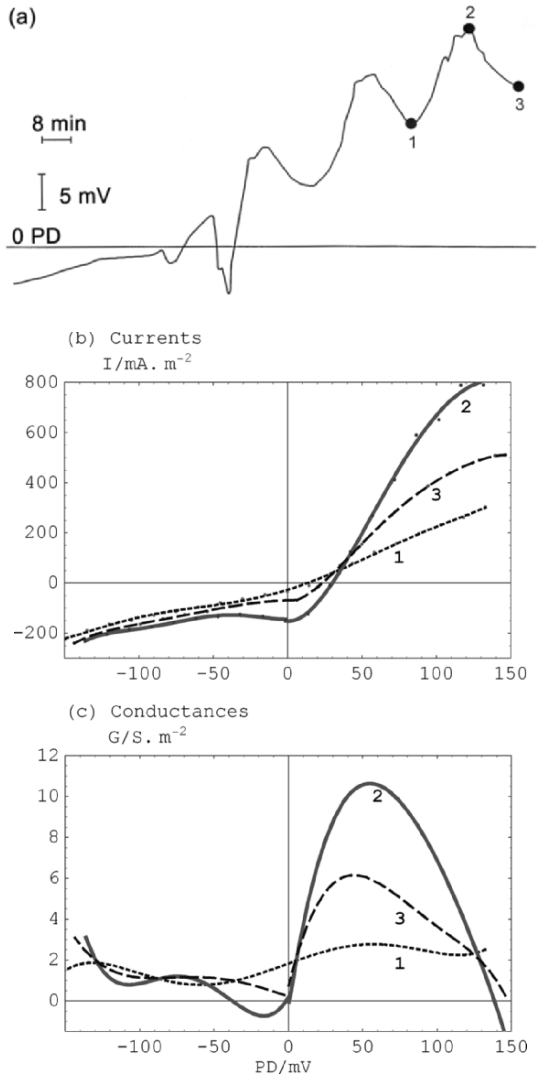

Fig. 16.3 K^+ pump oscillations in *Ventricaria* (extracted from Beilby and Bisson 1999). **a** Oscillations of membrane PD following cell impalement. **b** *I*/*V* characteristics at the crest (point 2) and the troughs (points 1 and 3) of the oscillations. **c** *G*/*V* characteristics obtained by differentiation of polynomials fitted to the *I*/*V* data

not reliable. The Cl^-, on the other hand, is probably imported through the $2H^+/Cl^-$ symporter (Beilby and Walker 1981), which will be investigated by removal of Cl^- from the outside medium. The modeling of the hypertonic regulation is in early stages, and demonstrates how the model can be built up gradually, while providing feedback for further experimental design.

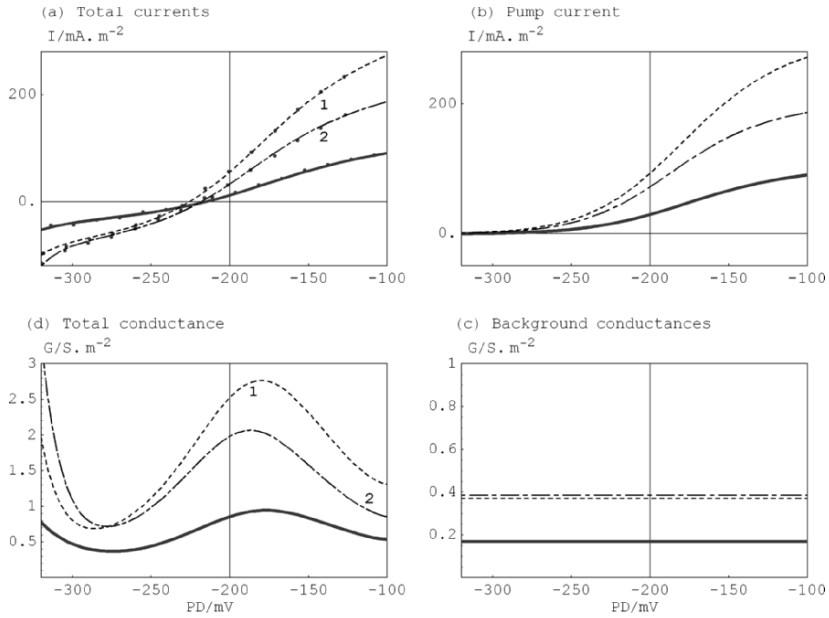

Fig. 16.4 Response of the salt-tolerant charophyte *Lamprothamnium* to hypertonic medium, step from 0.2 to 0.4 artificial seawater (Beilby and Shepherd 2001a). **a** Total current in steady state (*continuous line*), maximum current after 2 h 34 min in the hypertonic medium (*short dashed line*, curve 1), and diminishing current after 3 h 30 min in the hypertonic medium (*unequally dashed line*, curve 2). The data are shown as points. **b** Fitted pump currents (lines as in **a**). The parameters (in s^{-1}) are: steady state $k_{io}^0 = 2{,}800$, $k_{oi}{}^0 = 1.5$, $\kappa_{io} = 0.5$, $\kappa_{oi} = 55$, hypertonic curve 1 $k_{io}^0 = 11{,}000$, $k_{oi}{}^0 = 6.0$, $\kappa_{io} = 0.5$, $\kappa_{oi} = 160$, hypertonic curve 2 $k_{io}^0 = 9{,}900$, $k_{oi}{}^0 = 5.0$, $\kappa_{io} = 0.5$, $\kappa_{oi} = 108$. **c** Increase in G_{bkg} in hypertonic medium from 0.17 to 0.39 S m^{-2}. **d** Total conductances

16.3.2 Interaction of the Proton Pump and the Proton Channel in *Chara* spp.

If the *Chara* membrane is voltage-clamped to very negative levels (–400 to –500 mV) for about 12 s, the subsequent free-running membrane PD becomes strongly depolarized. The normal negative resting PD takes many seconds to recover. While there is a Cl^- efflux at the time of the negative PD clamp (Tyerman et al. 1986a, b), the after-depolarization is thought to be caused by the inhibition of the proton pump and activation of the H^+ channels (Beilby and Westermann, unpublished data). Similar effect could be observed in wheat root protoplasts, where the H^+ influx was measured by the MIFE system (Tyerman et al. 2001).

In Fig. 16.5, the cell exhibits a normal pump state (Fig. 16.5b; Beilby 1984). After being voltage-clamped to –490 mV for 12 s, *I*/*V* curves are data-logged at 2 min and 3 min after the end of the clamp. These *I*/*V* curves were fitted predominantly by H^+ current (Fig. 16.5c). The H^+ current was modeled by Goldmann-Hodgkin-Katz (GHK) equation (Eq.16. 3), multiplied by Boltzmann

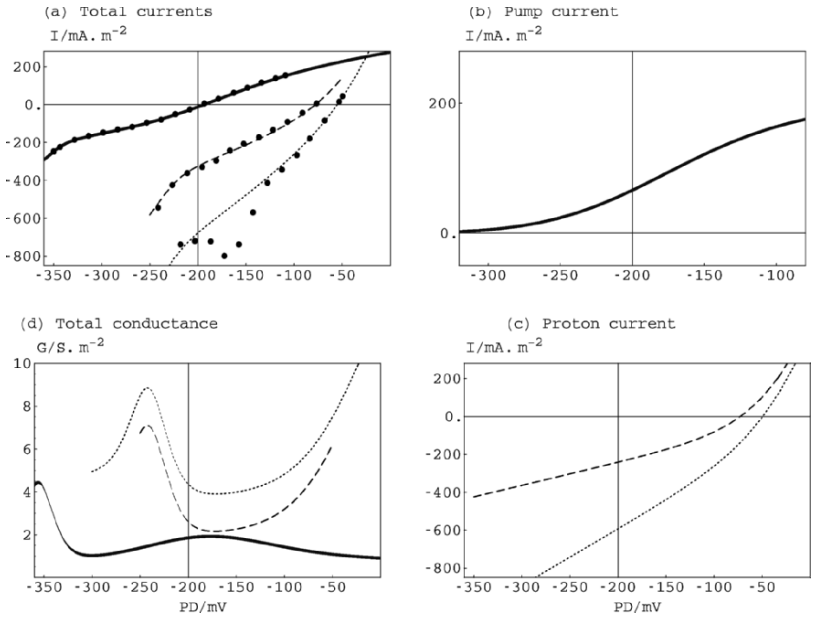

Fig. 16.5 Inactivation of the proton pump and activation of the proton channel by hyperpolarization. **a** Total currents before hyperpolarizing voltage clamp (*heavy continuous line*, data as points). Fit parameters (in s^{-1}): $k_{io}^{\ 0} = 3{,}400$, $k_{oi}^{\ 0} = 0.5$, $\kappa_{io} = 0.5$, $\kappa_{oi} = 105$, and a small contribution from the inward rectifier. At 2 min after the end of the voltage clamp to –490 mV for 12 s (*dotted line*, data as points). The value of pH_i was set at 6.65, that of pH_o at 7.5. *I/V* characteristics at 3 min after voltage clamp (*dashed line*, data as points). **b** The pump current prior to hyperpolarizing voltage clamp. **c** Proton currents. **d** Total conductances

distributions of open probabilities P_{o+} and P_{o-} (Eq. 16.3a, b) to simulate the channel closure, as the PD is clamped positive and negative of the channel closing thresholds (Beilby and Walker 1996; Amtmann and Sanders 1999).

$$I_X = \frac{P_{o+} P_{o-} N_X P_X (z_X F)^2 V \left([X]_i - [X]_o e^{-\frac{z_X FV}{RT}} \right)}{RT \left(1 - e^{-\frac{z_X FV}{RT}} \right)} \tag{16.3}$$

$$P_{o-} = 1 - \frac{1}{1 + e^{-\frac{z_g F(V - V_{50-})}{RT}}} \tag{16.3a}$$

$$P_{o+} = \frac{1}{1 + e^{-\frac{z_g F(V - V_{50+})}{RT}}} \tag{16.3b}$$

F, *R*, *T* have been defined above (see Eq. 16.2), z_X is the valence of the transported ion (H^+), *V* is the PD across the plasmalemma or across both

membranes, and $[X]_o$ and $[X]_i$ are the ion concentrations in the medium and the cytoplasm, respectively (in this case, pH_o and pH_i). $N_X P_X$ denotes the number of X (H^+) ion channels and their permeability and is treated as a single parameter, z_g is the number of gating charges, and V_{50-} and V_{50+} are the half-activation potentials at the negative and positive PD of channel closure, respectively.

The inward rectifier (K^+) was fitted by Eqs. (16.3) and (16.3a), with P_{o+} set to 1.0 (Beilby and Shepherd 2001a). The half-activation PD became depolarized in the post-clamp data (see conductance increase at −200 mV in Fig. 16.5d). The transient feature on the *I/V* curve at 2 min was not fitted. The simultaneous inactivation of the proton pump and activation of the H^+ channel is interesting, and hints at some level of interdependence between the transporters. This is also suggested by the responses to some inhibitors (Bisson 1986). The modeling is at an early stage.

16.4 Multiple Transporter Interaction

16.4.1 Hypotonic Regulation in Salt-Tolerant Charophytes

The hypotonic regulation involves a complex signal cascade, and the accompanying changes in membrane PD show a great variability (see Fig. 16.6 and review by Beilby et al. 2006). In this process, the *Lamprothamnium* cell has to get rid of ions from the vacuole to balance lowered water potential in the

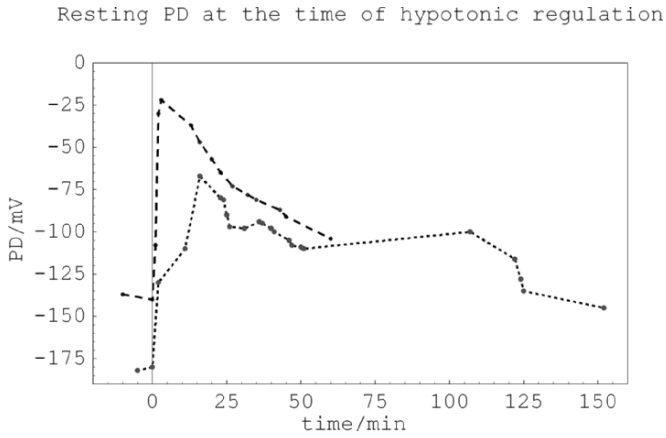

Fig. 16.6 The PD of two young *Lamprothamnium* cells (cell 1: *large points* and *dotted line*, cell 2: *smaller points*, *dashed line*) with minimal mucilage, undergoing hypotonic regulation after step from 1/3 to 1/6 artificial seawater (Beilby and Shepherd 1996). The hypotonic step was imposed at time 0. Note the PD hyperpolarization at 100 min, which indicates the reactivation of the proton pump

medium. This is a downhill process, where K^+, Cl^- and Na^+ flow out of the cell down their electrochemical gradients. How does the cell detect the increase in turgor? This is not known in any plant system. Our data suggest that at least some of the channels passing I_{bkg} are mechano-sensitive (Shepherd et al. 2002). Thus, the regulation is initiated by the MS channels as E_{bkg} depolarizes and G_{bkg} increases (Fig. 16.7d). The depolarization activates Ca^{++} channels either on plasmalemma, internal stores (Wacke and Thiel 2001), or both. The Ca^{++}-activated Cl^- channels dominate the first 20 min after the hypotonic exposure (Beilby and Shepherd 1996). The Cl^- channel *I*/*V* characteristics are inwardly rectifying (Fig. 16.7b; see also Beilby and Shepherd 2006a). If the cell is not in K^+ channel-dominated state prior to the hypotonic exposure, then the K^+ channels activate after about 10 min of the hypotonic stress (see Fig. 16.7c). How this activation occurs is not known. The rise in Ca^{++} concentration in the cytoplasm is not a likely trigger, as the activation was observed with the Ca^{++} increase blocked by La^{3+} (Beilby and Shepherd 1996).

The hypotonic response in *Lamprothamnium* is modulated by extracellular polysaccharide mucilage (Shepherd and Beilby 1999; Shepherd et al. 1999).

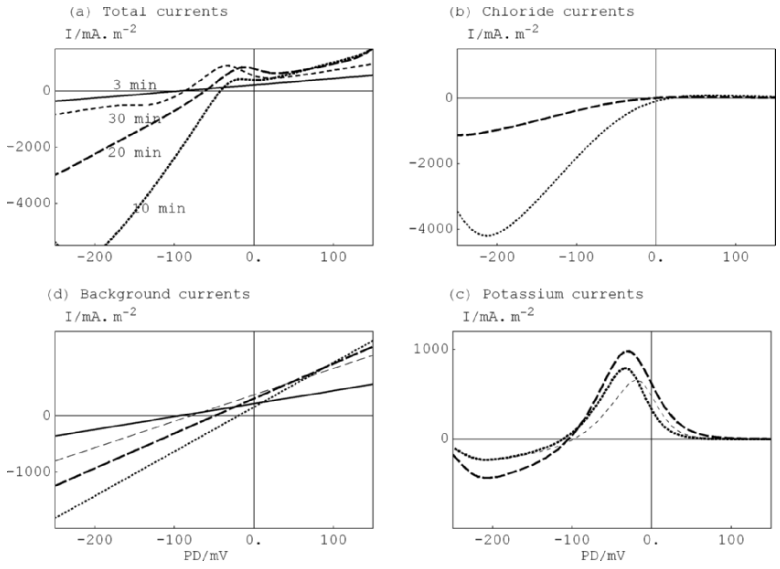

Fig. 16.7 a Synthesis of data from fitting hypotonic regulation under a range of conditions. All the data are for cells with minimal mucilage. The *continuous line* simulates the current at about 3 min after the hypotonic step, the *dotted line* 10 min, the *long dashed line* 20 min, and the *short dashed line* 30 min. **b** Cl^- current component at 10 and 20 min. The Cl^- currents were isolated by blocking the K^+ channels by TEA. For Eqs. (16.3) and (16.3a, b) parameters, see Beilby and Shepherd (2006a). **c** K^+ current component at 10, 20, and 30 min. The K^+ currents were isolated by blocking the Ca^{++} increase using La^{3+}. For Eqs. (16.3) and (16.3a, b) parameters, see Beilby and Shepherd (2001b). **d** The background currents were obtained by curve-fitting the data, from which the Cl^- and the K^+ currents were obtained

Young apical cells, with mucilage thickness below 5 μm, exhibited prolonged cessation of cytoplasmic streaming, and activation of both Cl^- and K^+ channels. The conductance increased by up to an order of magnitude, and the vacuolar K^+, Cl^- and Na^+ decreased to within 10% of the final value within 60 min (Beilby et al. 1999). The cells near the base of the plants or in high salinity formed thicker mucilage. As the mucilage thickness increased, the cells progressively lost activation of the Cl^- channels and K^+ channels, until only minimal response of the I_{bkg} remained. However, even these cells regulated their vacuolar ionic concentration over several days, confirming our hypothesis that I_{bkg} is carried by a mixture of ions.

Our model of the hypotonic regulation involves Ca^{++}-activated Cl^- channels, K^+ channels, and the background channels, set in a similar parallel circuit to that for the pump and background channels shown in Fig. 16.1a. At the time of hypotonic regulation, G_{Cl} is both PD and Ca^{++} concentration dependent, and E_{Cl} changes as chloride ions flow from the vacuole to the cytoplasmic compartment and out of the cell (Fig. 16.7b; Beilby and Shepherd 2006a). G_K is PD dependent, and E_K also changes as the regulation progresses (Fig. 16.7c; Beilby and Shepherd 2001b). Both G_{bkg} and E_{bkg} change, presumably as functions of the tugor pressure (Fig. 16.7d; Beilby and Shepherd 2006a). No currents were detected that could be attributed to Ca^{++} selective channels (see also Thiel et al. 1993), and so these were not included in the modeling. If the cell was in the pump state prior to hypotonic challenge, the pump appeared to be short-circuited and inhibited by the other rising conductances. In some cells, the pump reactivated about 2 h after the hypotonic challenge (Fig. 16.6).

The Cl^- and K^+ currents were also modeled by Goldmann-Hodgkin-Katz (GHK) equation (Eq. 16.3), multiplied by Boltzmann distributions of open probabilities P_{o+} and P_{o-} (Eq. 16.3a, b). The *I/V* analysis identified the sequence of background, Cl^- and K^+ channels becoming active throughout the regulation process. The modeling has also shown changes in the cytoplasmic concentration of Cl^- and K^+, as the ions move out from the cell. The K^+ channels on the plasmalemma might be activated by the increase in cytoplasmic K^+ concentration. Further work is planned to analyze the MS background channels, and their possible structural connections to the cell wall and cytoskeleton, which renders them sensitive to changes in turgor.

16.4.2 Repetitive Action Potentials in Salt-Sensitive Charophytes in High Salinity

In salt-sensitive *Chara* and salt-tolerant *Lamprothamnium*, the background conductance G_{bkg} increases in more saline media. The increase is greater if the Ca^{++} concentration of the medium is low (0.1 mM). A rise in pump activity counteracts this increase in G_{bkg} in *Lamprothamnium*, and the resting PD remains negative. The H^+ pump activity is crucial to provide both the pH gradient and the negative potential that enable K^+ and Cl^- import. The response of the *Chara* proton pump to increased salinity contrasts with that of

Lamprothamnium in surprisingly small, but complex ways. The resting PD in *Chara* becomes more positive in more saline media. This depolarization is due to the increase of Na^+ concentration, rather than a decrease in turgor pressure, since the cell PD and conductance hardly change in the presence of sorbitol (Beilby and Shepherd 2006b). Whereas the *Lamprothamnium* proton pump invariably increases its rate in more saline media, the *Chara* proton pump has more variable responses. Although some cells exhibit an increase in the same pump rate constants as was the case in *Lamprothamnium* (Beilby and Shepherd 2006b), the pumping rate is not sufficient to prevent membrane depolarization. The depolarization of the resting PD in *Chara* often leads to spontaneous action potentials. Similar behavior was observed in another salt-sensitive charophyte, *Nitella* (Kishimoto 1966). If the action potentials are stimulated, as in Fig. 16.8a, then the membrane PD does not recover and the action potentials recur spontaneously, often leading to strong depolarization and cell death. Low Ca^{++} causes greater depolarization (Fig. 16.8b), where the membrane PD takes a long time to recover.

As *Chara* and *Lamprothamnium* are closely related, a comparison of their electrophysiology under salinity stress will reveal the attributes that lead to extensive salt-tolerance of *Lamprothamnium*. This work is ongoing, but so far the main difference between the two charophytes is that the *Lamprothamnium* proton pump is activated by salinity increase, and the membrane PD becomes more negative and remains stable.

The repetitive action potentials are too fast to study by the *I*/*V* technique, but voltage clamp to a single PD level can be utilized to measure conductance changes. The instability of the *Chara* membrane PD in saline media is a vital clue to its salt-sensitivity and is currently subject of our investigations.

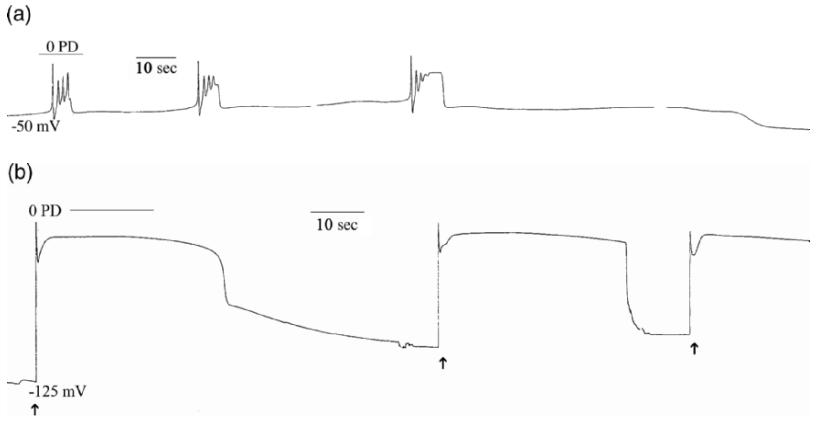

Fig. 16.8 Spontaneous action potentials and unstable membrane PD in *Chara* subjected to saline medium containing 100 mM NaCl and **a** 1.0 mM $CaCl_2$. The initial excitation followed the application of calibrated mechano-stimulator (Shepherd et al. 2001). **b** 0.1 mM $CaCl_2$: in this case, the action potential did not repeat spontaneously, but the membrane remained depolarized for many seconds. The times for each mechano-stimulation are shown as *arrows*

16.5 Conclusions

Changes in membrane PD provide vital clues to underlying signal cascades and transporter interaction, which enable the plant cell to maintain life-sustaining functions and respond to stress stimuli. Simultaneous *I*/*V* analysis and modeling allow the experimenter to identify the transporters involved, and to diagnose artifacts stemming from non-specificity of channel blockers and metabolic inhibitors. The models can be built up over time, starting from the most obvious *I*/*V* and *G*/*V* features and adding more subtle effects, as more information is gathered. For instance, the earlier study of K^+ state in *Chara* (Beilby 1985) facilitated the recognition of the K^+ state role in the hypotonic regulation in *Lamprothamnium* (Beilby and Shepherd 1996, 2001b). Most of the models of transporter interaction outlined in this chapter are still evolving, and will provide more information and future experimental design.

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